CHOLESTEROL METABOLISM IN RABBITS:
ITS VARIATION AND HERITABILITY

A thesis submitted to the

Australian National University

for the degree

of

Doctor of Philosophy

by

David Charles Keith Roberts

The Department of Experimental Pathology

John Curtin School of Medical Research

Canberra

June 1974

This thesis is my own work. Any collaboration with colleagues is indicated in the text.

D. C. K. Roberts

TABLE OF CONTENTS

	Page
List of Tables	vi
List of Figures	xi
Acknowledgements	xiii
Publications	xv
SUMMARY	xvi
INTRODUCTION	
1. Atherosclerosis	1
2. Therapies for atherosclerosis	2
3. Animal models for the study of atherosclerosis	3
4. The rabbit as an experimental model	4
5. Variations in plasma cholesterol in the rabbit	6
6. Cholesterol metabolism	9
(a) Absorption	9
(b) Transport	10
(c) Synthesis	13
(d) Catabolism	14
(e) Other factors in the control of cholesterol metabolism	15
7. Summary of cholesterol metabolism	16
MATERIALS AND METHODS	
1. Animals	19
2. Diets	19
3. Housing of animals and dietary consumption	20

4. Bleeding of animals 21

5. Chemicals 21

6. Chemical methods 22

(a) Cholesterol in plasma 22

(b) Cholesterol in feed 22

(c) Cholesterol and cholesteryl ester in liver, milk and plasma 23

(d) Phospholipid in plasma and milk 23

(e) Triglyceride in plasma and milk 24

(f) Protein in plasma, milk and hepatic microsomes 24

(g) Piperazine in feed 24

(h) Chromic oxide in feed and faeces 25

(i) Ascorbic acid in plasma, liver and adrenals 25

(j) Microsomal difference spectra 25

(k) Assay of radioactivity 26

7. Piperazine experiments in rabbits and guinea pigs 27

(a) Experimental design 27

(b) Preparation of microsomes 28

8. Rabbit breeding program 28

(a) Selection of breeding stock 28

(b) Experimental design 29

(c) Phenotyping of progeny 29

(d) Cross-fostering of progeny 30

(e) Sampling and analysis of rabbit milk 30

(f) Statistical analyses and estimation of heritability 30

9. Steroid balance experiments 31

(a) Animals and diets 31

(b) Experimental design 32

(c) Homogenisation and analysis of faecal materials 32

(d) TLC of neutral steroids 34

(e) Methylation and TLC of bile acids 35

(f) G.L.C. of neutral steroids and bile acid methyl esters 36

10. Analysis of plasma lipoproteins 38

(a) Experimental design 38

(b) Discontinuous density gradient ultracentrifugal method 38

(c) Classical ultracentrifugal method 40

(d) Immunoelectrophoresis 40

11. Enzyme assay experiments 41

(a) Animals 41

(b) Hepatic cholesterol synthesis 41

(c) Hepatic cholesteryl ester hydrolase 42

(d) Hepatic 7α-hydroxylase 43

(e) Plasma lecithin - cholesterol acyltransferase 47

RESULTS AND DISCUSSION 48

PART I. VARIATIONS IN CHOLESTEROL METABOLISM 48

1. Effect of piperazine on cholesterol metabolism in rabbits 48

(a) Results 48

(b) Discussion 54

2. Variation in plasma cholesterol concentration in non cholesterol-fed rabbits from the randomly outbred population 56

(a) Results 57

(i) Variation between sexes 57

(ii) Variation with age 58

(iii) Heritability 59

(b) Discussion 62

3. Variation in plasma cholesterol concentration in the cholesterol-fed rabbit 64

(a) Results 64

(i) Response to cholesterol feeding 64

(ii) Heritability of cholesterolaemic response 65

(b) Discussion 72

4. Influence of dam milk on progeny cholesterolaemic response 74

(a) Results 75

(b) Discussion 77

PART II. STUDIES ON HYPO-RESPONDER AND HYPER-RESPONDER RABBITS 79

1. Transport of cholesterol in the lipoproteins 79

(a) Results 79

(i) Validation of ultracentrifugal method 79

(ii) Analysis of plasma lipoproteins from non cholesterol-fed HO and HR rabbits 85

(iii) Analysis of plasma lipoproteins from cholesterol-fed HO and HR rabbits 88

(b) Discussion 92

2. Steroid balance study 93

(a) Results 95

(i) Plasma cholesterol concentration, specific radioactivity and body weight throughout the experiment 95

(ii) Steroid balance for HO and HR rabbits on a diet containing no added cholesterol 97

(iii) Steroid balance for HO and HR rabbits on a diet containing added cholesterol 103

(b) Discussion 109

3. Distribution of total cholesterol in HO and HR rabbits between plasma and liver and the relative contributions of cholesterol and cholesteryl ester 111

(a) Results and Discussion 112

4. Enzyme assays and tissue ascorbic acid analyses in HO and HR rabbits 116

(a) Results 117

(i) Body weight, liver weight and cholesterol concentrations in plasma and liver of HO and HR rabbits used for the enzyme experiments 117

(ii) Ascorbic acid in blood, liver and adrenals 118

(iii) Hepatic cholesterol synthesis 121

(iv) Hepatic cholesteryl ester hydrolysis 123

(v) Lecithin-cholesterol acyltransferase activity 125

(vi) Cholesterol 7α-hydroxylase activity 127

(b) Discussion 129

CONCLUSION 131

APPENDIX I

Determination of quench correction curves for liquid scintillation counting 139

REFERENCES 147

LIST OF TABLES

Table		Page
1	Methods for the assay of 7α-hydroxylase	44
2	Effects of dietary piperazine on plasma and liver cholesterol concentration in intact and castrated male and female cholesterol-fed rabbits	49
3	Effect of piperazine feeding on plasma cholesterol concentration in female rabbits	51
4	Hepatic microsomal cytochrome P-450 in intact and castrated cholesterol-fed rabbits and the effects of piperazine treatment	52
5	Hepatic microsomal cytochrome P-450 in cholesterol-fed guinea pigs and the effects of piperazine treatment	53
6	Mean initial plasma cholesterol concentration for male and female rabbits	57
7	Mean plasma cholesterol concentration of offspring	60
8	Components of variance of offspring plasma cholesterol concentration	61
9	Heritability of plasma cholesterol concentration in non cholesterol-fed rabbits	62
10	Mean cholesterolaemic response of parents and progeny of experimental matings in rabbits	66
11	Components of variance of progeny cholesterolaemic response	67
12	Mean cholesterolaemic response of full sibling progeny from first and second pregnancies	68

13	Effect of continued feeding of cholesterol diet on cholesterolaemic response of HO and HR rabbits	69
14	Heritability of the cholesterolaemic response by regression of progeny on parents	70
15	Heritability of the cholesterolaemic response by correlation of progeny	71
16	Analysis of milk from hypo-responder (HO) and hyper-responder (HR) rabbit dams	75
17	Mean plasma cholesterolaemic response of offspring suckled on natural and foster rabbit dams	76
18	Concentrations of the lipoprotein constituents of plasma from a hypercholesterolaemic rabbit	81
19	Distribution of the lipoprotein constituents within the lipoprotein species of plasma from a hyper-cholesterolaemic rabbit	82
20	Presence of precipitin lines in rabbit lipoprotein fractions after immunoelectrophoresis	83
21	Presence of precipitin lines to specific anti-sera in human lipoprotein fractions after immunoelectrophoresis	84
22	Concentrations of the lipoprotein constituents of plasma from non cholesterol-fed HO and HR rabbits	86
23	Distribution of lipoprotein constituents within the lipoprotein species of plasma from non cholesterol-fed HO and HR rabbits	87
24	Concentrations of the lipoprotein constituents of plasma from cholesterol-fed HO and HR rabbits	90

38	Free cholesterol and cholesteryl ester concentrations in the livers of HO and HR rabbits - cholesterol-fed	115
39	Body weights, liver weights and plasma and liver cholesterol concentrations of HO and HR rabbits used for the enzyme experiments	119
40	Ascorbic acid concentrations in the tissues of cholesterol-fed HO and HR rabbits	120
41	Incorporation of 1-^{14}C-acetate by liver slices in cholesterol-fed HO and HR rabbits	122
42	Hepatic cholesteryl ester hydrolase activity in cholesterol-fed HO and HR rabbits	124
43	LCAT activity in non cholesterol-fed and cholesterol-fed HO and HR rabbits	125
44	Concentrations of free cholesterol in the incubation mixture for the assay of LCAT activity in non cholesterol-fed and cholesterol-fed HO and HR rabbits	126
45	Activity of cholesterol 7α-hydroxylase in rat and rabbit	128
46	Focal program MCID used to calculate the matrix coefficients for solving the equations of the quench curves for liquid scintillation counting	142
47	Focal program SMID used to calculate the coefficients of the quench curves used in liquid scintillation counting	143
48	Calculation of coefficients of ^{3}H quench curve using programs MCID and SMID	144

49	Data files of coefficients for quench curves produced by different agents or scintillation mixtures	145
50	General Focal program, L21D for determining radioactivity (as mμCi) under conditions of variable quenching and with dual label counting	146

LIST OF FIGURES

Figure		Between pages:
1	Schematic representation of mammalian cholesterol metabolism	17-18
2	Effect of piperazine on hypercholesterolaemia in castrated male and castrated female rabbits	48-49
3	Frequency distribution of plasma cholesterol concentration of male and non-pregnant, non-lactating female rabbits	57-58
4	Regression of the mean plasma cholesterol concentration of non cholesterol-fed male rabbits on age	58-59
5	Regression of mean progeny plasma cholesterolaemic response on mid-parent response	72-73
6	Immunoelectrophoresis of rabbit lipoproteins isolated by ultracentrifugation	83-84
7	Immunoelectrophoresis of human lipoproteins isolated by ultracentrifugation	84-85
8	Concentrations of lipoprotein constituents within density classes of plasma from hypo-responder rabbits	88-89
9	Concentrations of lipoprotein constituents within density classes of plasma from hyper-responder rabbits	89-90
10	Plasma cholesterol concentrations of hypo- and hyper-responder rabbits	95-96

11	Plasma cholesterol specific radioactivity of hypo- and hyper-responder rabbits	96-97
12	Chromatogram of the trimethylsilyl derivatives of rabbit acidic steroid methyl esters separated by GLC	98-99
13	Daily cholesterol balances in hypo-responder (HO) and hyper-responder (HR) rabbits consuming a diet containing no added cholesterol	102-103
14	Rate of cholesteryl ester hydrolysis in rabbit liver	123-124
15	Activity of plasma lecithin-cholesterol acyltransferase in rabbits	125-126

ACKNOWLEDGEMENTS

The work reported in this thesis would not have been possible without the assistance of many people. In particular, I should like to thank Professor F. C. Courtice for giving me the opportunity to undertake this work and for his encouragement throughout my research. I also wish to thank my supervisor Dr C. E. West for the many occasions on which his valued help and constructive criticism enabled my work to proceed. In addition, I should like to thank Dr T. G. Redgrave[1], with whom I collaborated on the development of the ultracentrifuge method, for his helpful advice and assistance during the formative months of my work. My thanks are also due to Dr J. B. Smith for providing the space and maintaining the necessary facilities for the breeding of the selected rabbits. Dr M. W. Simpson-Morgan[2] and Mr H. R. Kinns were of great assistance with statistical and computer programming problems. Dr J. B. Coombe[3] willingly provided the facilities for the preparation of the special diets and Dr W. R. Sobey[4] provided helpful advice on the construction of the breeding program.

---

[1] Department of Physiology, University of Melbourne, Parkville, Vic. Formerly of this department.

[2] Department of Animal Husbandry, University of Queensland, St Lucia, Queensland. Formerly of this department.

[3] C.S.I.R.O. Division of Plant Industry, Canberra.

[4] C.S.I.R.O. Division of Animal Genetics, North Ryde, N.S.W.

Technical assistance was of the highest calibre. Mr J. R. Harding and Mr I. L. Flanagan ensured that equipment and stores were readily available and Mrs M. M. Lee, Mrs D. J. Rich and Mrs D. C. Sutherland were patient, helpful and efficient typists. I also wish to acknowledge the skilful assistance given at various times by Miss A. J. Pomroy, Miss S. M. Smith, Mrs B. Tan and Mr O. R. Bryant.

Finally, I should like to thank my wife, without whose assistance, patience and forbearance, this thesis would not have been possible.

Certain sections in this thesis have been published.

WEST, C. E., T. G. REDGRAVE and D. C. K. ROBERTS. Effect of castration on cholesterol metabolism and hepatic cytochrome P-450 in piperazine-treated male and female rabbits. Aust. J. exp. Biol. med. Sci. 52, 185-192 (1974).

ROBERTS, D. C. K., C. E. WEST, T. G. REDGRAVE and J. B. SMITH. Plasma cholesterol concentration in normal and cholesterol-fed rabbits: its variation and heritability. Atherosclerosis 19, 369-380 (1974).

ROBERTS, D. C. K. and C. E. WEST. The heritability of plasma cholesterol concentration in the rabbit. Heredity (in press).

ROBERTS, D. C. K. and C. E. WEST. The influence of rabbit milk on the cholesterolaemic response of the offspring of rabbits. Lipids (in press).

REDGRAVE, T. G., D. C. K. ROBERTS and C. E. WEST. Separation of plasma lipoproteins by density gradient ultracentrifugation. Analyt. Biochem. (in press).

SUMMARY

During the course of studies on the effect of piperazine on cholesterol metabolism, considerable inter-animal variation was noted and the study of this variation became the main theme of this thesis.

The observations of Redgrave and West (1972) on the differential effect of piperazine on the cholesterol-aemia produced by cholesterol feeding in male and female rabbits were extended to castrated male and female rabbits. Castration did not alter the differential effect of piperazine on cholesterol metabolism, i.e. in male rabbits hypercholesterolaemia was reduced but in females it was augmented.

In the same experiments hepatic microsomal cytochrome P-450 was measured. Intact female rabbits had higher P-450 concentrations than males and the difference was removed after castration. Piperazine reduced P-450 concentrations in males but not females.

It was concluded that the differential effect of piperazine on cholesterol metabolism in male and female rabbits must operate at a chromosomal or somatic level of sexual differentiation and not through the gonad. There is no simple relationship between hepatic total P-450 concentration and cholesterol metabolism.

To study the wide variation noted during the above experiments, a large population of normal rabbits was examined. The plasma cholesterol concentration of this population showed a wide range of variation primarily associated with sex, age, season and inheritance.

The plasma cholesterol concentration:

a) is higher in females than males

b) decreases with age in males and is unchanged in females

c) shows greater seasonal variation in females than males

d) is lower in pregnant and lactating females than in non-pregnant, non-lactating females

e) has a heritability of 62±38.8% in females and 22±20.2% in males.

The large error in the male estimate of heritability makes it difficult to state if the trait is heritable in male rabbits.

Studies of a large population of cholesterol-fed rabbits showed a wide range of cholesterolaemia. The cholesterolaemia produced by cholesterol feeding:

a) shows a positive correlation for both males and females between the initial plasma cholesterol concentration and the increase observed after three weeks on a diet containing added cholesterol

b) is similar for both sexes

c) is heritable

d) may decrease with age in both males and females.

A controlled breeding trial from selected hyper-responding and hypo-responding parents established that the cholesterolaemic response to dietary cholesterol has a heritability of 50±4.7%. It is suggested that the transmission of the character for cholesterolaemia is polygenic.

The milk lipids from the dams of the two strains of rabbits differing in their cholesterolaemic response; one hyper-responsive (HR) and one hypo-responsive (HO) to

dietary cholesterol, were analysed. The milk from HR dams had significantly higher ($P<0.05$) cholesterol and phospholipid concentrations than that from HO dams but similar triglyceride concentrations. Cross-fostering experiments with HO and HR offspring were carried out. Offspring from HO parents suckled on HR dams resembled HR offspring in their cholesterolaemic response. However, offspring from HR parents responded as HR whether they were raised on their natural dams or on foster HO dams. It is concluded that the trait for hyper-responder characteristics is uninfluenced by rabbit milk whilst the trait for hypo-responder characteristics is dependent on the cholesterol and/or phospholipid concentrations in milk.

Further studies were conducted on the HO and HR rabbits to determine the biochemical causes for the difference in the cholesterolaemia produced by cholesterol feeding.

A method was developed in collaboration with Dr T. G. Redgrave for the separation of plasma lipoproteins into very low density - (VLDL), low density - (LDL) and high density lipoprotein (HDL) by a single 24-hour ultracentrifugation in swinging bucket rotors. The method employs a discontinuous salt gradient and separates VLDL, LDL and HDL as verified by a classical method and immuno-electrophoresis. Its use for the analysis of human plasma lipoproteins was demonstrated.

HO and HR rabbits, when fed the cholesterol containing diet differ in their plasma cholesterol concentration, liver cholesterol concentration and total

faecal steroid output. It has been demonstrated that cholesterol-fed HO rabbits when compared with cholesterol-fed HR rabbits have:

a) lower plasma cholesterol concentration
b) higher liver cholesteryl ester concentration and similar liver free cholesterol concentration
c) lower ratio of free cholesterol to esterified cholesterol in the liver
d) similar percent distribution of cholesterol, triglyceride, phospholipid and protein within the various plasma lipoproteins
e) similar plasma lecithin-cholesterol acyl transferase activity
f) similar hepatic cholesteryl ester hydrolase activity
g) similar suppression of hepatic cholesterol synthesis
h) similar ascorbic acid concentrations in blood adrenals and liver
i) increased daily output of total faecal steroids derived from cholesterol.

HO and HR rabbits do not differ in any of the above ways when fed the diet containing no added cholesterol.

It is concluded that cholesterol feeding is necessary to demonstrate the difference between HO and HR rabbits and this difference is associated with the plasma/liver partition of cholesterol and the ability of the HO animals to excrete more total faecal steroids. This may be either by decreased absorption or increased endogenous output.

INTRODUCTION

1. Atherosclerosis

Atherosclerosis is a major cause of death and debility in present day society (Epstein, 1973) and yet despite many years of research its aetiology has still not been fully elucidated. It is a complex process which may be regarded as a dynamic interaction among (a) the structural and metabolic properties of the arterial wall, (b) the components of the blood and (c) the haemodynamic forces (Getz, Vesselinovitch and Wissler, 1969). The ultimate result of such factors is a narrowing of the lumen of the vessel. Complete occlusion may follow thrombus formation or haemorrhage into the affected arterial wall. Involvement of a coronary artery may lead to a coronary event. To the more familiar "risk factors" associated with atherosclerosis and coronary heart disease (CHD) such as hypertension, hypercholesterolaemia, hypertriglyceridaemia, obesity, smoking and physical activity (Eades, 1968; Morris and Gardner, 1969), have been added in recent epidemiological studies various other components such as heredity (Rose, 1969), mental stress (Rosenman and Friedman, 1971) and water hardness (Rose, 1969). Unexplained variability is still present in a population after all the other risk factors have been taken into consideration (Strong and Eggen, 1969). Thus it can be seen that there are numerous predisposing factors to atherosclerosis many of which in fact may only be secondarily related to the production of CHD. A primary causative agent has not yet been demonstrated, that is,if the disease is not multifactorial. The original proposition that

atherosclerosis was a viral disease of the aorta has recently been reintroduced and discussed by Cliff (1974) who suggests that the disease has only recently reached epidemic proportions because of population pressures. With this multiplicity of evidence for the aetiology of atherosclerosis it is not surprising that the researcher has turned to animal models. These provide not only several advantages but also certain obvious disadvantages as discussed below.

2. Therapies for atherosclerosis.

As hyperlipoproteinaemia is one of the major risk factors (National Institutes of Health, 1971) and is more readily determined than the presence of atherosclerotic lesions, therapy has come to depend on inference from the hyperlipaemic state. This usually concentrates on reducing the hyperlipaemia, usually the hypercholesterolaemia, by diet and/or drugs on the assumption that this will reverse or halt the progress of the disease. At the same time, other risk factors are treated and reduced if possible. It is generally believed that those people regarded as being at risk should be on diets in which the fat component contains a significant proportion of mono-unsaturated and poly-unsaturated fats (Brown, 1969). However there is considerable controversy over the large scale substitution of polyunsaturated fats for saturated fats in the population at large (West and Redgrave, 1974). In all the above treatments, the primary aim is to reduce plasma cholesterol concentration on the assumption that this will halt or

retard the progress of atherosclerosis (Eades, 1968; Dayton, 1971). For testing hypocholesterolaemic agents animal models are necessary and such models are discussed in the next section.

3. Animal models for the study of atherosclerosis.

The advantages of animal models can be summarised as follows:

experimental atherosclerosis can be produced in a comparatively short time;

dietary and environmental factors can be controlled;

a variety of animal models allow for the collection of complementary data;

pre-clinical testing of various drugs for the reduction, prevention or reversal of atherosclerosis can be carried out;

hypotheses as to the aetiology of atherosclerosis can be tested.

However, there are major disadvantages to animal models. The first is the obvious one that the results obtained in animals may not be validly applied to man. The second is that a really good model for human atherosclerosis has yet to be found, although swine and sub-human primates closely approximate the human condition (Clarkson, 1972). The third disadvantage is that dietary manipulation, usually involving increased lipid intake, is necessary for the production of atherosclerosis. The incidence of spontaneous atherosclerosis in rabbits, chickens, turkeys, pigeons, swine and sub-human primates has been reviewed by

Clarkson (1963) and by Stout and Groover (1969). The production of dietary induced atherosclerosis in various laboratory animals and sub-human primates has been reviewed by Kritchevsky (1969) and Clarkson (1972).

In all animal models with spontaneous atherosclerosis, hypercholesterolaemia produced by dietary cholesterol exacerbates the lesion (Clarkson, 1972). In addition, atherosclerotic lesions have been induced by hypercholesterolaemia in species which do not naturally develop the lesions (Clarkson, Pritchard, Bullock, Lehner, Lofland and St. Clair, 1970). Despite the differences in the lesions produced in animals, the involvement of hypercholesterolaemia in the production of atherosclerosis in both man and animals provides encouragement for the use of animal models.

4. <u>The rabbit as an experimental model</u>.

The rabbit was the first animal to be used as a model for atherosclerosis (Saltykow, 1908; Ignatowski, 1909) and despite certain disadvantages it has continued to be used for such purposes. The suitability of the rabbit as a model has been reviewed by Clarkson (1963, 1972) and Kritchevsky (1969). Briefly, the principal advantage has been the rapidity with which the animal becomes hypercholesterolaemic and hence atherosclerotic when fed a variety of natural or semi-synthetic diets. Cholesterol or casein fed as a component of normal rabbit feed produces hypercholesterolaemia (Carroll, 1971). Saturated fat enhances the hypercholesterolaemia and polyunsaturated fat reduces it, although these agents added alone to normal

rabbit food do not produce hypercholesterolaemia (Kritchevsky and Tepper, 1968; Carroll, 1971). Semi-synthetic diets without added cholesterol produce hypercholesterolaemia which is enhanced by saturated fat and reduced by polyunsaturated fats (Carroll, 1971). The carbohydrate and protein source of the diet also influence the degree of hypercholesterolaemia produced (Kritchevsky, Sallata and Tepper, 1968; Meeker and Kesten, 1941). The disadvantages of the rabbit as a model, centre upon the types and distribution of atherosclerotic lesion produced by dietary manipulation. Prior, Kurtz and Ziegler (1961) have contrasted the distribution of lesions in the human with those found in the cholesterol-fed rabbit. However, with suitable manipulation of the animal through dietary and hormonal means, lesions resembling those of the human type can be produced (Constantinides, Booth and Carlson, 1960; Constantinides, 1961; Clarkson et al., 1970). The objections to studying "cholesterol storage disease" in the rabbit can be overcome by producing atherosclerotic lesions with a cholesterol-free semi-synthetic diet (Kritchevsky, 1969). Further support for the use of the rabbit as a model for the study of atherogenesis has appeared recently when it was shown that rabbits use the same method for the transport of lipid into atherosclerotic plaques as occurs in human aortic lesions (Walton, 1973). However, as in man, there is considerable inter- and intra-strain variations in the susceptibility of rabbits to both hypercholesterolaemia and atherosclerosis. It is this variation in hyper-cholesterolaemia in rabbits which is the subject of this

thesis. An understanding of the causes of variation in cholesterol metabolism in the rabbit may help in elucidating the wider problem in man.

5. Variations in plasma cholesterol in the rabbit.

There are many references in the literature to variations in the plasma cholesterol concentration in rabbits. For example Laird, Fox, Schultz, Mitchell and Blau (1970) observed considerable differences between eleven strains of rabbits, and Wang, Schaeffer, Drachman and Adlersberg (1953) within three strains. Differences between sexes have also been observed - male rabbits have been shown to have significantly lower plasma cholesterol concentrations than female rabbits (Laird et al., 1970; Fillios and Mann, 1956).

Variations with age have also been shown. Shope (1928) showed that for male rabbits after weaning, there was a gradual decline in plasma cholesterol concentration from 45 days to 292 days. For females there was a more erratic decline with the concentration being higher than that of the males at similar time points. However only 1 male and 2 female rabbits were studied. More recently, Laird and Fox (1970) have shown a rapid decrease post-weaning, for both sexes of rabbits, with a much less pronounced decrease from 50-120 days. Similarly, Geison and Waisman (1970), in studies on male rabbits demonstrated a decrease in plasma cholesterol concentration from 56 to 126 days.

The cholesterolaemic response of rabbits to dietary cholesterol has been known and studied ever since

Anitschkow (1914) fed cholesterol containing diets to rabbits and produced lipid accumulations in the aortic intima. Variations in the magnitude of the hypercholesterolaemia produced by cholesterol feeding for varying periods up to 12 months have been reported by Rohrschneider (1925), Thölldte (1927), Turner and Bidwell (1937) and Carroll (1971). These variations can be grouped into 3 or 4 types of cholesterolaemic response (Pollak, 1945; Sinitsina and Lovyagina, 1956) and Fillios and Mann (1956) suggested classifying rabbits as hypo-responders, normo-responders or hyper-responders to dietary cholesterol. There are many reports of rabbits resistant to dietary induced hypercholesterolaemia (Deicke, 1926; Turner, Present and Bidwell, 1938; Fillios and Mann, 1956; Sinitsina and Lovyagina, 1956; Novitskii, 1971). Where the sex of the animals studied is mentioned, this resistance is more common in males than females (Turner and Bidwell, 1937; Fillios and Mann, 1956) and occurs with an incidence of between 10 and 20% of animals studied. The above reports on resistance to the development of hypercholesterolaemia were based on results from various strains of rabbits using various methods for the administration of the cholesterol to the animal. The magnitude of the cholesterolaemia produced by feeding amorphous or crystalline cholesterol, or cholesterol suspended in corn oil is quite variable (Kritchevsky, Marcucci, Sallata and Tepper, 1969). In general, increased quantities of cholesterol fed per day produces increased cholesterolaemia (Fillios and Mann, 1956; Adams, Gaman and Feigenbaum, 1972). However, the degree of atherosclerosis

produced is more dependent on the vehicle used to administer the cholesterol than on the increase in plasma cholesterol concentration (Clarkson, 1963). Rabbits resistant to cholesterol-induced hypercholesterolaemia have been reported in the New Zealand White, Chinchilla and Dutch belted strains (Fillios and Mann, 1956; Novitskii, 1971; Turner et al., 1938; Adams et al., 1972). In this laboratory, hypo-responder rabbits of our outbred semi-lop strain were first observed during a study of the differential effects of piperazine on the cholesterolaemia produced by cholesterol feeding in male and female rabbits (Redgrave and West, 1972). Further work on piperazine was carried out as described below but large groups of animals were necessary because of the appearance of these resistant animals. It was therefore decided to study this variation because there was an indication from the breeding records of the rabbits in the colony maintained by the Animal Breeding Establishment of the Australian National University, that the variation might be inherited. Thus there was the possibility of selecting for a strain of rabbits with a reproducible cholesterolaemic response to dietary cholesterol. This would be an extremely useful group for the testing of hypocholesterolaemic agents. In this thesis is reported the results of a breeding program for the selection of hyper- and hypo-responder rabbits and the heritability of such characteristics. Further experiments are reported determining the biochemical differences in cholesterol metabolism between the two groups. A discussion of

cholesterol metabolism and its control is contained in the next section.

6. Cholesterol metabolism.

Cholesterol metabolism and its regulation has been extensively reviewed (Goodman, 1965; Dietschy and Wilson, 1970a, b, c; Ho and Taylor, 1970; Rautureau, Coste and Rautureau, 1972) and only a brief summary of the essential features will be mentioned here.

(a) Absorption:

The role of the enterohepatic circulation has been reviewed recently by Dowling (1972) and is intimately involved in cholesterol and fat absorption (Watt and Simmonds, 1970; Simmonds, 1969). Exogenous cholesterol ester is hydrolysed in the presence of bile-salt activated pancreatic cholesterol ester hydrolase. If sufficient bile acids, fatty acids and monoglycerides are present the free cholesterol is solubilized in mixed micelles (Dietschy and Wilson, 1970a) and brought into contact with the brush borders of the intestinal absorptive cells. Maximal absorption of cholesterol takes place in the proximal region of the jejunum (Borgström, 1960) and is presumed to be a passive process (Taylor and Ho, 1967). Once absorbed the cholesterol mixes with the intracellular pool of free cholesterol, some of which will be derived from synthesis in situ. Most of this is esterified, incorporated in chylomicra, and released into the intestinal lymphatics (Rautureau et al., 1972). Regulation of absorption depends on bile salts being present and whether fat is fed with the cholesterol (Deuel, 1955). Fat in the diet leads to

increased absorption of cholesterol. Whether this is a result of the fat stimulating bile flow, providing additional fatty acids and monoglycerides for micelle formation, or increasing chylomicron formation or all three is not known (Dietschy and Wilson, 1970b). The size of chylomicrons and hence their capacity for cholesterol transport is also affected by the amount of fat fed (Fraser and Courtice, 1969). Unsaturated fats result in better absorption of cholesterol than saturated fats (Gould and Cook, 1958) and considerable variation between species occurs in the ability to absorb cholesterol (Rautureau et al., 1972).

Once absorbed, cholesterol contained in lymph chylomicra enters the blood stream via the thoracic duct. Lipoprotein lipase rapidly removes triglyceride at tissue sites and the cholesterol eventually reaches the liver contained in chylomicron remnant particles (Redgrave, 1970) where it undergoes various metabolic processes associated with the lipoproteins.

(b) Transport:

All the major lipids in blood are transported predominantly as lipoproteins. Reviews on the apoprotein and lipid moieties of the lipoproteins (Peeters, 1970), on plasma lipid transport systems (Lopez-s, 1971) and on the metabolism of the plasma lipoproteins (Lewis, 1972) have appeared recently. In this section I shall only discuss the lipoproteins with reference to cholesterol metabolism. Since preparative ultracentrifugation was established as a method for separating plasma lipoproteins into chylomicrons, very low density - (VLDL), low density - (LDL) and high

density-lipoproteins (HDL) (Lindgren, Elliot and Gofman, 1951; Havel, Eder and Bragdon, 1955), increasing sophistication of techniques has produced an ever increasing number of sub-classes of these major density classes. Cholesterol is a component of all lipoproteins with the major proportion being present as cholesterol ester (Peeters, 1970). There are considerable species differences in the density class which carries the majority of the cholesterol (Mills and Taylaur, 1971). As mentioned above, cholesterol contained in chylomicrons, mainly as the ester, is rapidly transported to the liver as remnant particles (Redgrave, 1970) after triglyceride has been removed at tissue sites. There is a delay of some hours before this cholesterol re-emerges from the liver in the circulating lipoproteins (Goodman, 1965). This would support the hypothesis of Sodhi and Kudchodkar (1973) which suggests that there are two pools of cholesterol within the liver; an anabolic pool to which exogenous and newly synthesised cholesterol contribute and from which the lipoprotein cholesterol is derived: a catabolic pool to which lipoprotein and mobilised tissue cholesterol contribute and from which bile acids and cholesterol are excreted.

LDL and HDL are produced in the liver and perhaps in the small intestine (Lewis, 1972) and may also be generated as the end products of chylomicron and VLDL metabolism (Lopez-s, 1971). Fidge and Foxman (1971) have shown, in the rat, that LDL is formed from delipidated VLDL. Endogenous triglyceride is transported by VLDL which is produced in the liver (Schumaker and Adams, 1969). The

regulation of lipoprotein structure during the removal and addition of components to circulating lipoprotein is thought to involve the maintenance of the cholesterol: cholesterol ester ratio within the lipoproteins. The enzyme, lecithin-cholesterol acyltransferase (LCAT) catalyses the esterification of plasma cholesterol with the concomitant conversion of lecithin to lysolecithin (Glomset, 1968). This enzyme is closely associated with HDL and preferentially esterifies HDL cholesterol (Lossow, Shah and Chaikoff, 1966). Glomset (1968) has proposed that LCAT is important in the transport of free cholesterol from tissues to the liver for catabolism. He suggests that free cholesterol enters the plasma, is incorporated into HDL, esterified by LCAT and may then exchange with the other lipoproteins before reaching the liver. Schumaker and Adams (1969) have suggested a further role for LCAT and have presented the following overall scheme for lipoprotein metabolism. Chylomicrons and VLDL, on entering the circulation, acquire apoprotein A from HDL or VLDL which facilitates the action of LCAT and lipoprotein lipase. Triglyceride is removed from VLDL and chylomicrons and at the same time LCAT removes cholesterol and lecithin, maintaining the integrity of the particles. The lysolecithin produced enters HDL and the cholesterol ester may enter LDL or HDL for subsequent transport to the liver. The more recent work of Redgrave (1970) has shown that the cholesteryl ester-rich chylomicron remnant particle is rapidly transported to the liver where the cholesterol is removed.

(c) Synthesis:

Cholesterol synthesis takes place in most tissues of the body but the only quantitatively important sites are the liver and intestine (Dietschy and Wilson, 1970a). The rate limiting enzyme of cholesterol synthesis has been shown to be β-hydroxy-β-methylglutaryl coenzyme A reductase (HMG-CoA reductase) which catalyses the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid (Slakey, Craig, Beytia, Briedis, Feldbrugge, Dugan, Qureshi, Subbarayan and Porter, 1972). The same authors suggest that under conditions of cholesterol feeding, the enzyme squalene synthetase may become rate limiting. The entero-hepatic circulation of bile acids is responsible for the regulation of liver cholesterol synthesis. Bile acids act indirectly, as the absorption of cholesterol is dependent on the quantity of bile acids in the intestinal tract. It is this exogenous cholesterol which inhibits hepatic cholesterol synthesis at the HMG-CoA reductase step (Ho and Taylor, 1970). The bile acids have also been shown to act directly on HMG-CoA reductase (Hamprecht, Nüssler, Waltinger and Lynen, 1971; Hamprecht, Roscher, Waltinger and Nüssler, 1971). Starvation inhibits hepatic cholesterol synthesis by a mechanism as yet unclear (Ho and Taylor, 1970; Dietschy and Wilson, 1970a). Intestinal cholesterol synthesis is directly inhibited by bile salts at the HMG-CoA reductase step, but not by exogenous cholesterol. Thus intestinal synthesis becomes an important component when dietary cholesterol consumption is high.

Diurnal variations in hepatic cholesterol synthesis have been demonstrated in the rat and mouse (Back, Hamprecht and Lynen, 1969; Horton, Hickman and Sabine, 1970; Kandutsch and Saucier, 1969). The control of this diurnal fluctuation in the rat is due to the synthesis of new HMG-CoA reductase enzyme which only occurs for six hours per day (Higgins, Kawachi and Rudney, 1971).

(d) Catabolism:

The catabolism of cholesterol to the primary bile acids, 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxycholanic acids takes place in the liver and the regulatory step is the initial 7α-hydroxylation of cholesterol (Daniellson, Einarrson and Johansson, 1967). This enzyme, cholesterol 7α-hydroxylase is microsomal and cytochrome P-450 dependent (Wada, Hirata, Nakao and Sakamoto, 1968; Trülzsch, Greim, Czygan, Hutterer, Schaffner, Popper, Cooper and Rosenthal, 1973). Side chain cleavage of the hydroxylated cholesterol takes place in the mitochondria (Van Belle, 1965). Before excretion into bile, the bile acids are conjugated mainly with taurine and/or glycine. There are considerable species variations in the ratio of taurine to glycine conjugates found in bile and this has been linked to the susceptibility of the species to develop atheroma (Van Belle, 1965; Kritchevsky, 1969).

Cholesterol is excreted directly into the bile, and together with the conjugated bile acids, enters the intestinal tract. An appreciable quantity of the faecal neutral steroid output is derived from the cholesterol of the desquamated mucosal cells (Dietschy and Wilson,

1970c; Lewis, 1972). The bile acids and cholesterol undergo further biotransformations in the intestinal tract. Bacterial action releases the bile acids from their conjugates and also produces the secondary bile acids, 3α-hydroxycholanic acid and 3α,12α-dihydroxycholanic acid. These may be reabsorbed and recycled through the entero-hepatic circulation and thus bile acid output in the bile consists of a mixture of primary and secondary conjugated bile acids. Cholesterol is principally transformed to 5β-cholestan-3β-ol and 5β-cholestan-3-one by the intestinal flora (Dietschy and Wilson, 1970c). Cholesterol is also catabolised to hormonal products but this is not a major contributor to cholesterol turnover.

(e) Other factors in the control of cholesterol metabolism:

Other factors involved in the control of cholesterol metabolism are cytochrome P-450, ascorbic acid and hormones all of which have been reviewed recently (Gibbons and Mitropoulos, 1973; Ginter, 1972; Myant, 1968). Cytochrome P-450 is the enzyme complex involved in hydroxylation reactions requiring molecular oxygen and is responsible for drug detoxication in the liver. The same enzyme complex is reported to be associated with the biosynthesis of cholesterol from lanosterol (Hirata, Wada and Sakamoto, 1967; Wada, Hirata and Sakamoto, 1969; Gibbons and Mitropoulos, 1973) and the catabolism of cholesterol to 7α-hydroxycholesterol (Wada et al., 1968; Trülzsch et al., 1973; Atkin, Palmer, English, Morgan, Cawthorne and Green,

1972). The role of ascorbic acid in cholesterol metabolism is confusing. It appears to increase the oxidation of cholesterol especially in the hypercholesterolaemic state (Ginter, 1972) and may mobilise cholesterol from the tissues (Spittle, 1971). Hormones are involved in the control of both the anabolism and catabolism of cholesterol. Synthesis is increased by thyroid hormones and oestrogens and inhibited by androgens (Myant, 1968). Thyroid hormone also stimulates the removal of cholesterol as neutral sterol and stimulates the conversion to bile acids (Myant, 1968). Corticosterone affects the diurnal variation in synthesis (Hickman, Horton and Sabine, 1972). Noradrenalin increases synthesis (Bortz, 1968) and insulin and glucagon increase the activity of HMG-CoA reductase (Huber, Guder, Latzin and Hamprecht, 1973). The conversion of cholesterol to steroid hormones is also under hormonal control (Myant, 1968).

7. Summary of cholesterol metabolism.

Thus, dietary cholesterol is mixed with cholesterol from bile and the intestinal mucosa and absorbed into the intestinal wall as free cholesterol in the form of mixed micelles. Here, together with some locally synthesised cholesterol, it is esterified and incorporated into chylomicron particles, enters the lymph, is discharged into the bloodstream via the thoracic duct and after loss of triglyceride in the tissues reaches the liver contained in chylomicron remnant particles. In the liver, together with the endogenously synthesised cholesterol it can be (a) catabolised to bile acids, (b) excreted in the bile,

(c) incorporated into the lipoproteins. However, Sodhi and Kudchodkar (1973) would suggest that only (c) would occur and that only returning lipoproteins and mobilised tissue cholesterol would undergo (a) and (b). The excreted bile acids enter the enterohepatic circulation and (a) act in the formation of micelles for the absorption of cholesterol, (b) regulate intestinal cholesterol synthesis, (c) affect liver cholesterol synthesis. Figure 1 illustrates schematically the current concepts of the regulation of cholesterol metabolism as described above.

In this section I have attempted to define the areas to be studied in cases of disturbed or altered cholesterol metabolism. In summary, production or reduction of hypercholesterolaemia whether by drugs, diet or familial means can be produced by:

1. Altered absorption of exogenous cholesterol.
2. Altered synthesis of cholesterol.
3. Altered transport of cholesterol in the lipoproteins.
4. Re-distribution of cholesterol among the tissue, liver and plasma pools.
5. Altered catabolism of cholesterol to bile acids.
6. Altered excretion of cholesterol as faecal neutral steroids.
7. Altered enterohepatic circulation of bile acids (affects 1, 2 and 4).

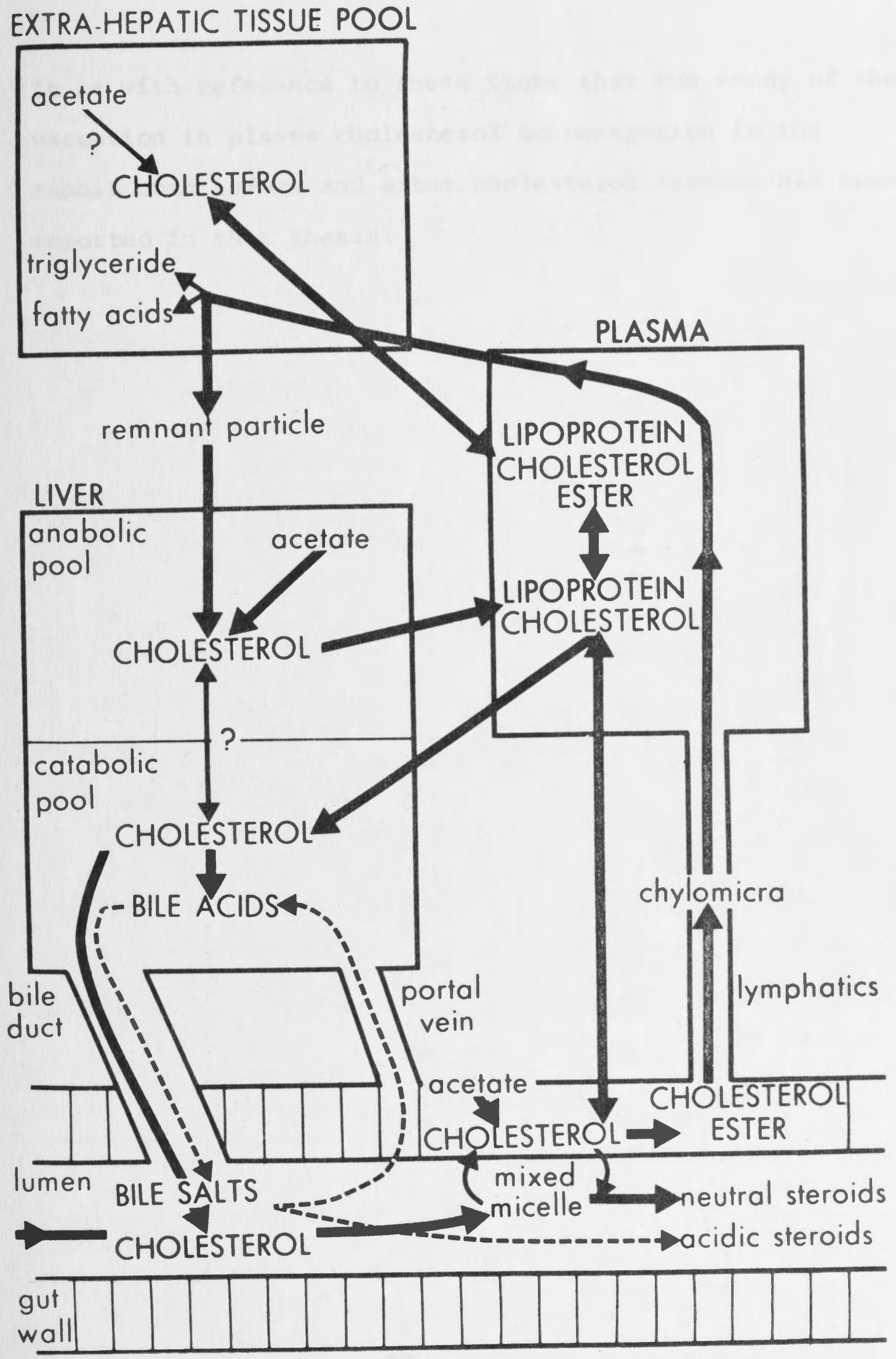


Figure 1. *Schematic representation of mammalian cholesterol metabolism.*

It is with reference to these items that the study of the variation in plasma cholesterol concentration in the rabbit both before and after cholesterol feeding has been reported in this thesis.

MATERIALS AND METHODS

1. Animals.

All animals used were from colonies maintained in the Animal Breeding Establishment of the Australian National University. The rabbits were a semi-lop strain which had been developed by random outbreeding from a nucleus of New Zealand white rabbits with introductions of full-lop, wild and Californian strains. Animals for the breeding program were selected from this colony as described below. The guinea pigs were from an albino outbred stock.

2. Diets.

Full details of diets used in the experiments described in this thesis are given because inadequate provision of data has often made it difficult to interpret or reproduce other workers' findings (Pollak, 1965). The source of the basic feed has varied throughout the experimental series. The rabbit feed for the piperazine experiments was supplied in pellet form by Allied Feeds, Rhodes, N.S.W., 2138, and had the following proximate composition: crude protein 20%, crude fat 4 to 5% and crude fibre maximum 7.5%. The Allied feed was ground before adding cholesterol or piperazine. For the rabbit breeding and subsequent experiments with rabbits and guinea pigs, the feed was formulated by Mecon Agricultural Products, Eastwood, N.S.W., 2122, and had the following proximate composition: crude protein 18.0%, crude fat 3 to 3.5% and crude fibre maximum 10.0%. The feed was compounded and supplied in mash form, initially by Wm. Connelly and Son, Goulburn, N.S.W., 2580, and later by Bunge Australia Pty Ltd,

Murrumburrah, N.S.W., 2595. For the breeding experiment, the diet with added cholesterol was initially supplied as pellets by Wm. Connelly and Son but later, cholesterol was incorporated in the diet in this laboratory. Cholesterol and other additives were uniformly mixed with the diets using a cement mixer, and pellets (½" long) were prepared with a Provender Templewood Jr. pelletting machine fitted with a 5/32" die (Lister Blackstone Pty Ltd, Revesby, N.S.W., 2212). Commercial grade cholesterol supplied by Townson and Mercer Pty Ltd, Lane Cove, N.S.W., 2066, was used in the diets at a concentration of 0.28% (w/w) corrected for impurities. Piperazine citrate (B.P.C., VS Supplies Ltd, Camperdown, N.S.W., 2050) was added to the diet at a concentration of 0.56% (w/w calculated as anhydrous piperazine). For the sterol balance experiment, chromic oxide was added to the diet at a concentration of 0.12% (w/w). Cholestyramine (4% w/w, Cuemid:Merck, Sharp and Dohme, West Point, Pa., 19486, U.S.A.) was added to the cholesterol-containing diet for some of the experiments in which 7α-hydroxylase was measured.

3. Housing of animals and dietary consumption.

Rabbits maintained in the breeding colony and used in the breeding program were housed up to 4 in a cage and allowed free access to the basic diet. The rabbits used in all experiments were housed individually and received 70 g of feed per day.

Guinea pigs were housed six to a cage and allowed free access to all diets. The guinea pigs' water was supplemented daily with 0.010% (w/v) L-ascorbic acid

(analytical grade) providing approximately 20 mg per guinea pig per day.

All animals were fed daily at 9 a.m. or after sampling of blood and allowed access to water at all times.

4. Bleeding of animals.

Rabbits were bled from the marginal ear vein, before feeding, into tubes containing EDTA (final concentration 4 mM, pH 7.4) as anti-coagulant. Plasma was separated from the chilled blood by centrifugation. Guinea pigs were bled by heart puncture and the blood was collected and separated as described above. At the termination of experiments not involving enzyme assays rabbits were anaesthetized i.v. and guinea pigs i.p. with Nembutal (pentobarbitone sodium 60 mg/ml Abbott Laboratories Pty Ltd, Kurnell, N.S.W., 2230, 0.5 ml/kg) and blood collected from the inferior vena cava. In enzyme assay experiments the animals were killed either by a blow to the head or by an i.v. injection of air.

5. Chemicals.

Hexadecane (1,2-^{3}H), hexadecane (1-^{14}C), acetate (1-^{14}C), cholesterol (7α-^{3}H), cholesterol (1,2-^{3}H) and cholesterol (4-^{14}C) were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Cholic acid (^{3}H-G) and cholesterol (4-^{14}C) oleate were obtained from New England Nuclear Corp., Boston, Mass., 02118, U.S.A. Chromic oxide was supplied by E. Merck, 61 Darmstadt, Germany. 3α-hydroxy-, 3α,12α-dihydroxy- and 3α,7α,12α-trihydroxy-cholanic acids and their methyl esters were obtained from Steraloids Inc., Pawling, N.Y., 12564, U.S.A. 3α,6α-dihydroxy cholanic acid was

obtained from Fluka A.G. Buchs S.G., Switzerland. Cholest-5-ene-3-one was obtained from BDH Ltd, Poole, England, and 5α-cholestane from Sigma Chemical Co., St Louis, Miss., 63118, U.S.A. From the cholest-5-ene-3-one two reference substances were prepared by Dr C. E. West. 5β-cholestan-3-one was prepared by reduction with palladium catalyst in ether (Grasshof, 1934a) and from this material, 5β-cholestan-3β-ol was obtained by reduction with platinum black in acetic acid (Grasshof, 1934b). Cholesterol used as standard in the cholesterol assay was purified via the dibromide (Fieser, 1953) from laboratory reagent grade supplied by BDH. The o-phthaldialdehyde reagent was puriss grade and supplied by Fluka. Trimethylchlorosilane was supplied by Hopkin and Williams Ltd, Chadwell Heath, England, and hexamethyldisilazane was obtained from Pierce Chemical Co., Rockford, Illinois, 61105, U.S.A. All radiochemicals except hexadecane were purified by preparative TLC prior to use.

6. Chemical methods.

(a) Cholesterol in plasma:

The method of Mann (1969) was used for the hydrolysis and extraction of cholesterol. Cholesterol in the petroleum ether extract was measured using the o-phthaldialdehyde colour reagent described by Zlatkis and Zak (1969).

(b) Cholesterol in feed:

5 g of feed was heated under reflux with 130 ml of 0.77 N KOH in 77% (v/v) aqueous ethanol for 2 hours.

After cooling, the mixture was shaken with exactly 50.0 ml of petroleum ether (b.p. 60-80°C) and then with 40 ml of water. Then after suitable dilution the cholesterol concentration of the petroleum ether phase was measured as described below.

(c) Cholesterol and cholesteryl ester in liver, milk and plasma:

The tissue was extracted and non-lipid contaminants removed by the method of Folch, Lees and Sloane Stanley (1957). An aliquot was taken and chromatographed on thin layers (0.5 mm) of Silica gel G on glass plates (20 x 20 cm) to separate cholesterol and cholesteryl ester, with petroleum ether (b.p. 60-80°C):diethyl ether:glacial acetic acid (70:30:2 by volume) as eluting solvent. The lipid bands were visualised under u.v. light using Ultraphor (BASF Australia Ltd, Sydney, N.S.W., 2017, 0.01% w/v in water). The cholesterol and cholesteryl ester bands were each scraped into sintered glass funnels (porosity 3) and eluted with 30 ml of petroleum ether (b.p. 60-80°C):diethyl ether (1:1 v/v). The cholesteryl ester was hydrolysed and extracted as described above. The unesterified cholesterol and the cholesterol from the cholesteryl ester were assayed as described above.

(d) Phospholipid in plasma and milk:

Folch-washed lipid extracts were separated into neutral lipid and phospholipid fractions by the method of Zilversmit (1965) on columns of silicic acid-kieselguhr. Silicic acid was supplied by Bio-Rad Laboratories, Richmond, Calif., U.S.A. and kieselguhr (Hyflo-Supercel) by Townson

and Mercer Pty Ltd, Lane Cove, N.S.W., 2066. In some experiments the kieselguhr was omitted as adequate flow rates could be achieved with the silicic acid (SilicAR CC-7) supplied by Mallinckrodt Chemical Works, St Louis, Miss, U.S.A. The separation of neutral lipids and phospholipids was checked by TLC. Lipid phosphorous was measured by the turbidimetric method of Eibl and Lands (1969).

(e) Triglyceride in plasma and milk:

Glyceride glycerol of the neutral lipid fraction was estimated by the spectrophotometric method of Zilversmit (1965).

(f) Protein in plasma, milk and hepatic microsomes:

The protein precipitate from the Folch extract of milk or from the lipoproteins (described below) was dissolved in sodium hydroxide (1N). Aliquots from these protein solutions and from hepatic microsomal suspensions were taken for estimation of protein nitrogen by direct nesslerization of Kjeldahl digests (Minari and Zilversmit, 1963).

(g) Piperazine in feed:

A sample of feed (1 g) was shaken for two hours on a mechanical shaker with exactly 100 ml of water. After centrifugation at 400g for 10 minutes an aliquot was removed for colorimetric estimation using Folin's amino acid reagent based on the method described by Rogers (1958).

(h) Chromic oxide in feed and faeces:

Known weights of feed (approx. 5 g) or homogenised faeces and urine (25-30 g) were evaporated to dryness at 110°C in porcelain evaporating basins. The samples were then transferred to a muffle furnace and ashed at 600°C for one hour. In order to prevent loss of sample material when the basins were placed in the muffle furnace, the initial temperature was not greater than 100°C. The chromic oxide content of the samples was then determined as Cr^{+++} essentially as described by Williams, David and Iismaa (1962) using an atomic absorption spectrophotometer (Model AA-4, Varian-Techtron Pty Ltd, North Springvale, Victoria, 3170).

(i) Ascorbic acid in plasma, liver and adrenals:

Ascorbic acid was assayed as dehydroascorbic acid coupled to 2,4-dinitrophenylhydrazine by the method of Schaffert and Kingsley (1955). Whole blood was extracted with TCA and tissues were homogenised in TCA (final concentration 4% v/v TCA). Ascorbic acid in the supernatant was oxidised with activated charcoal (BDH) and assayed as described by Schaffert and Kingsley (1955).

(j) Microsomal difference spectra:

The content of cytochrome P-450 was determined spectrophotometrically as described by Omura and Sato (1964). An aliquot of the microsomal suspension was diluted 1 in 10 with 0.1M phosphate buffer (pH 7.0). Solid sodium dithionite (1-2 mg) was added and the suspension was then divided and placed in two cuvettes of 1 cm light path. Carbon monoxide was bubbled through the sample cuvette for

twenty seconds. The difference spectrum between the sample and reference cells was recorded from 600 nm to 400 nm using a recording spectrophotometer (Unicam model SP 8000, Pye Unicam Ltd, Cambridge, England). The molar extinction coefficient for cytochrome P-450 was taken as 91 $mM^{-1}cm^{-1}$ and the results expressed as µmoles per g microsomal protein as described by Omura and Sato (1964).

Substrate-induced difference spectra were determined as described by Mitani and Horie (1969). Equal aliquots (3.0 ml) of the diluted microsomal suspension were placed in two cuvettes. Aliquots of 1mM steroid dissolved in 1,2-propanediol were added to the sample cuvette and a similar amount of 1,2-propanediol were added to the reference cuvette. Water soluble compounds were added in 0.1M phosphate buffer, pH 7.0 or water. The difference spectra were recorded as described above.

(k) Assay of radioactivity:

A liquid scintillation counter was used (model LS100 Beckman Instruments Inc., Fullerton, Calif., 92634, U.S.A.). Solvent was evaporated under nitrogen from the samples contained in counting vials. Coloured samples were decolourised by the addition of a methanolic solution of hydrogen peroxide. Once decolourised, the solvent was evaporated and scintillator (10.0 ml) consisting of diphenyloxazole (PPO) in toluene (0.5% w/v) was added. Digitonide precipitates were dissolved in methanol (1.0 ml) and added to the above scintillator (10.0 ml). Quench correction curves were constructed using ^{3}H- and ^{14}C-hexadecane. A computer program was written using these

quench correction curves to calculate the spillover of ^{14}C counts into the ^{3}H channel and to correct for variable quenching (see Appendix 1). The program was written in Focal programming language for use with a PDP8/i computer (Digital equipment Co., Maynard, Mass., 01754, U.S.A.).

7. Piperazine experiments in rabbits and guinea pigs.

(a) Experimental design:

For each experiment, rabbits from the general colony were paired on the basis of their initial plasma cholesterol concentrations and body weight, and allocated one to each group in order to minimise inter-group variations.

Rabbits were castrated at 8-12 weeks of age. Anaesthesia was induced using sodium thiopentone (Intraval Sodium, May and Baker Ltd, Footscray West, Victoria, 3012, 0.25%, 1 ml/kg body weight) and maintained on either methoxyflurane (Penthrane Abbott Laboratories) or anaesthetic ether. After orchidectomy or ovariectomy the rabbits were kept for 8 weeks before the commencement of the experiment.

The animals were fed 70 g per day. The daily ration contained 200 mg cholesterol and one group also received 400 mg piperazine (expressed as the anhydrous base). In some experiments only piperazine (400 mg) was fed in the daily ration.

The plasma cholesterol concentration was measured at weekly intervals. On the last day of the experiment the rabbits were anaesthetised with pentobarbitone sodium (Nembutal 60 mg/ml, Abbot Laboratories, 0.5 ml/kg) and

the liver was perfused in situ via the portal vein with saline (0.9% w/v, 200 ml) at room temperature after cutting the supradiaphragmatic inferior vena cava. The liver was removed, blotted dry, weighed and samples were taken for the analysis of cholesterol and the preparation of microsomes.

(b) Preparation of microsomes:

A representative sample (10 g) of liver was homogenized in 4 volumes of ice cold KCl (1.15% w/v) by four strokes of a Potter-Elvehjem teflon-glass homogeniser. The homogenate was centrifuged in a 50 ml swinging bucket rotor (model HB4) in a centrifuge (model RC2, Ivan Sorvall Inc., Norwalk, Conn, U.S.A.) at 8,700 rev/min (12,000g) for 20 minutes at 0°C to remove cell debris and mitochondria. The supernatant was centrifuged at 27,000 rev/min (105,000g) for 75 minutes in an ultracentrifuge (Model L3-50, Beckman Instruments Inc. Spinco Division, Palo Alto, Calif, U.S.A.) using a SW 27 rotor with 6x40 ml buckets. The microsomal pellet was resuspended in 1.15% KCl with a Dounce homogeniser and recentrifuged at 105,000g for 75 minutes. The washed microsomal pellet was then suspended in 0.1M phosphate buffer (pH 7.0, 10.0 ml), to give a concentration of about 15 mg protein per ml.

In the experiments by Mr S. D. Turley on the effect of piperazine in guinea pigs, liver was removed and microsomes prepared for the determination of cytochrome P-450 as described for rabbits.

8. Rabbit breeding program.

(a) Selection of breeding stock:

The plasma cholesterol concentration was

measured in all animals between 8 and 20 weeks of age in the breeding colony. The rabbits were then ranked according to these concentrations. Rabbits from the highest and lowest ten percentiles of cholesterol concentrations were selected for further study since a correlation between the plasma cholesterol concentration and the degree of cholesterolaemia produced by cholesterol feeding has been established (see below). The selected rabbits were then fed 70 g per day of the cholesterol containing diet (200 mg cholesterol per day) for a period of three weeks, at which time their plasma cholesterol concentration was again determined. On the basis of these results, hypo-responding (HO) and hyper-responding (HR) rabbits were chosen for use in the subsequent breeding program.

(b) Experimental design:

The dams were not used for mating until at least six weeks had elapsed from the end of cholesterol feeding. At this time the plasma cholesterol concentration had returned to the pre-cholesterol feeding concentration. The progeny were removed from the dams six weeks after birth, the males separated from the females, caged and then fed the basic diet (without added cholesterol).

(c) Phenotyping of progeny:

At ten weeks of age, the plasma cholesterol concentrations of the progeny were determined. They were then caged individually and fed the cholesterol diet for three weeks after which the plasma cholesterol concentration was again determined. The progeny were phenotyped

according to their cholesterolaemic response. Wherever possible subsequent breeding pairs were selected from among the progeny of the experimental matings.

(d) Cross-fostering of progeny:

Within two days of birth, young from matings of HR males and females were cross-fostered with those from matings of HO males and females. The young were phenotyped as described above.

(e) Sampling and analysis of rabbit milk:

Ten days post partum the dams were milked manually. Oxytocin (Pitocin, Parke Davis Co., Sydney, N.S.W., 1 Unit/kg) was given intravenously via the marginal ear vein prior to milking. An aliquot of the milk was analysed for the concentration of protein, triglyceride, cholesterol and phospholipid as described in the chemical methods section.

(f) Statistical analyses and estimation of heritability:

Heritability (h^2) is the ratio of the genetic variance to the total (genetic plus phenotypic) variance and may be estimated in a number of ways. Where both the parental and progeny values were known, more than one method of estimating the heritability was used. This was so for the estimation of the heritability of the cholesterolaemic response. Regression of progeny response on parental response, as described by Falconer (1963), provided one estimate of heritability. Correction for unequal litter sizes was made by using a weighting factor (w_n) calculated from the phenotypic correlation of siblings

within families (Falconer, 1963). The regression coefficient so obtained, when multiplied by two, provided an estimate of the heritability. Analysis of variance for the effect of litter size, sex and parents on the cholesterolaemic response of the progeny was also determined and the various components tested for significance.

Heritability was also estimated by the paternal half-sibling method described by Falconer (1963). This was the only method used for estimating the heritability of the plasma cholesterol concentration in non cholesterol-fed rabbits as the parental values were not known. Briefly, this involves an analysis of variance leading to the estimation of three components of variance, attributable to sires (between half-sib families) to dams (between dams within sires) and to individuals (within dams). These values are equated with the expected composition of the variances which are dependent on the number of offspring per litter and per half-sib family. From these equations the correlation between half-sib families can be obtained and this, multiplied by four, provides an estimate of the heritability.

9. Sterol balance experiment.

(a) Animals and diets:

HR and HO rabbits aged between 20 and 30 weeks were used. These animals were the progeny from the breeding program and had been phenotyped as HR or HO by feeding the cholesterol diet as described in Section 8(c). They were not used for at least six weeks after cholesterol feeding at which time their plasma cholesterol concentration had

returned to their pre-cholesterol feeding concentration. All diets contained chromic oxide (0.14% w/w) as an inert marker to correct for faecal losses. One diet also contained cholesterol (0.28% w/w). Each animal received 70 g of diet per day containing a known amount (about 100 mμC) of 4-^{14}C-cholesterol (S.R.A. 80.54 mCi/mM). The isotope, dissolved in ethanol, was added to the daily ration the evening before it was to be given to the animal to allow the ethanol to evaporate before feeding.

(b) Experimental design:

The animals received the cholesterol-free diet for eight weeks. Plasma cholesterol concentration was measured weekly. An aliquot of the petroleum ether phase containing cholesterol was transferred to a counting vial, the solvent removed by evaporation under nitrogen and the sample assayed for radioactivity. From five weeks onwards, weekly faecal collections were made for analysis of neutral and acidic steroids. Faeces and urine were collected together on plastic covered trays beneath the wire base of the individual rabbit cages. Whilst most of the faeces dropped through the wire, sufficient remained for the normal practice of coprophagy to take place. Dietary material was carefully excluded from the faecal samples. At eight weeks the animals received the cholesterol-containing diet for a further five weeks. Weekly plasma cholesterol measurements and faecal collections were continued for this period.

(c) Homogenisation and analysis of faecal materials:

The combined faeces and urine were homogenised,

with the addition of water, to a smooth consistency in a Waring blendor (Waring Products Co., Winsted, Conn, U.S.A.). Aliquots were taken for the determination of chromium (Section 6(h)) and for the extraction of neutral steroids (Miettinen, Ahrens and Grundy, 1965) and acidic steroids (Grundy, Ahrens and Miettinen, 1965). A known amount (25-30 g) of the homogenate was placed in a centrifuge bottle with a ground glass neck (250 ml) to which was added absolute ethanol (100 ml), 10N KOH (10 ml) and three glass beads. Tritiated cholic acid (^{3}H-G) and cholesterol (7α-^{3}H) were added for calculation of recoveries of acidic and neutral steroids. The mixture was then heated under reflux for two hours on a boiling water bath. After cooling, distilled water (40 ml) was added and the neutral steroids extracted with 3 x 50 ml of petroleum ether (b.p. 60°-80°C). The petroleum ether of the pooled extract was evaporated under reduced pressure on a rotary evaporator at 37°C. The residue containing the neutral steroids was dissolved and made up to 50.0 ml in petroleum ether (b.p. 60°-80°C). To the aqueous phase containing the acidic steroids was added 10N KOH (10 ml) and the contents heated under pressure at 15 psi in a pressure cooker for three hours to hydrolyse the bile acid conjugates. After cooling, the mixture was acidified to pH 2 by the careful addition of concentrated HCl. Filter aid (Hyflo-Supercel, Townson and Mercer Pty Ltd, 10 g) was added and the mixture filtered through filter paper (Whatman No. 541) under reduced pressure into a centrifuge bottle with a ground neck (500 ml). The original centrifuge bottle was

rinsed with chloroform:methanol (2:1 v/v, 50 ml) and this was poured through the filter paper to extract any steroids in the precipitate. The bottle containing the filtrate was shaken and then centrifuged at 1000g for five minutes. A tapered glass tube (O.D. 8-10 mm) filled with chloroform was carefully lowered through the upper aqueous phase and the lower phase was quantitatively aspirated into a round bottom flask (250 ml). The aqueous phase was extracted twice more with chloroform (50 ml). The solvent of the pooled lower phase was evaporated under vacuum on a rotary evaporator and the residue containing the bile acids was dissolved and made up to 50.0 ml with chloroform: methanol (2:1 v/v).

To determine the recovery of neutral and acidic steroids, aliquots were taken from both extracts for assay of radioactivity.

(d) TLC of neutral steroids:

An aliquot of the neutral steroid extract was separated by preparative TLC on florisil (Floridin Co., Hancock, W.Va., U.S.A.0 as described by Miettinen et al. (1965) into three fractions in descending order:

I 5β-cholestan-3-one and 3-keto derivatives of plant sterols

II 5β-cholestan-3β-ol and ring saturated 5β derivatives of plant sterols

III Cholesterol, campesterol, stigmasterol, β-sitosterol and corresponding ring saturated 5α sterols

The fractions were eluted with diethyl ether, the solvent evaporated under nitrogen and a known amount of 5α-cholestane added as an internal standard prior to GLC.

(e) Methylation and TLC of bile acids:

An aliquot of the bile acid extract was evaporated under nitrogen, the residue dissolved in dry methanol (0.5 ml) and methylated by the addition of an ethereal solution of diazomethane prepared by the method of Werner (1919). The methylated bile acids were separated from fatty acid methyl esters and other contaminants by chromatography on thin layers (0.5 mm) of alumina (E. Merck) on glass plates (20x20 cm). The solvent systems used were those described by Grundy et al. (1965) except that toluene was substituted for benzene. The methyl esters of the fatty acids and bile acids were visualised under u.v. light using Ultraphor (0.01% w/v in water). Alumina provided a similar separation to that obtained with silica gel H by Grundy et al. (1965) without the need for a preliminary development of plates to remove contaminants contained in the gel. The gel containing the bile acid methyl esters was scraped into sintered glass funnels (porosity 3) and the solutes eluted under reduced pressure with methanol (25 ml). Alumina has the further advantage that unlike silica gel, it is not soluble in methanol and thus does not block the sinter. The solvent of the bile acid methyl ester sample was evaporated under nitrogen and the residue dissolved and made to 10.0 ml with methanol. An aliquot was taken for gas-liquid chromatography and a known amount of 5β-cholestan-3β-ol added as internal standard.

(f) G.L.C. of neutral steroids and bile acid methyl esters:

Samples were analysed on a gas chromatograph (Series 1800, Varian Aerograph, Walnut Creek, Calif., 94598, U.S.A.) equipped with a variable temperature programmer module and with dual hydrogen flame ionization detectors. Two stainless steel columns length 3 metres, O.D. 1/8 inch) were packed with Gas Chrom Q (100-120 mesh, Applied Science Laboratories Inc., State College, Penn., U.S.A.) coated with 3% methyl silicone (JXR, Applied Science Laboratories) installed and conditioned as described by Vandenheuval and Court (1968). Nitrogen was used as carrier gas at a flow rate of 60 ml/min. The air flow rate was 300 ml/min and the hydrogen flow rate 60 ml/min. The injector temperature was 270°C and the detector temperature 290°C. Samples for injection onto the columns were dissolved in carbon disulphide (neutral steroids) or carbon disulphide: acetone (1:1 v/v, bile acids) and aliquots (1-3 µl) placed on a solid injector (Model Sl-1, Scientific Glass Engineering Pty Ltd, North Melbourne, Victoria, 3051) using a micro-syringe (Model 5-A-R N-G-P., Scientific Glass Engineering Pty Ltd). After the solvent had evaporated the samples were injected onto the column.

The free hydroxyl groups of the neutral steroids were trimethylsilylated by the method of Miettinen et al. (1965). An aliquot of the neutral steroid extract was taken and after evaporation of solvent the residue was dissolved in dry chloroform:hexamethyldisilazane (9:1 v/v,

100 µl) and trimethylchlorosilane (100 µl) was subsequently added. The reaction mixture was left at room temperature for one hour after which time the solvents were evaporated under nitrogen and the residue dissolved in carbon disulphide for GLC. The column oven temperature was maintained at 240°C throughout the elution. The peak height and the retention time relative to the internal standard 5α-cholestane were measured to determine the quantity of the individual neutral steroids. Quantitation by this technique has been shown to be reproducible (C. E. West, personal communication).

The free hydroxyl groups of the bile acid methyl esters were trimethylsilylated by the method of Grundy et al. (1965) using dry pyridine:hexamethyldisilazane: trimethylchlorosilane (9:3:1 by volume) and the reaction mixture left at room temperature for a minimum of two hours. The solvent was then evaporated under nitrogen and the residue dissolved in carbon disulphide:acetone (1:1 v/v) for GLC. The column oven temperature was programmed as follows:

1. Injection at 180°C increasing at 15°/min for four minutes.
2. Isothermal at 240°C for four minutes and then increasing at 6°/min for four minutes.
3. Isothermal at 264°C for 36 minutes and then increasing at 4°/min for four minutes.
4. Isothermal at 280°C.

The internal standard and the bile acid derivatives were eluted during the 264°C isothermal period. The areas under the peaks were measured by planimetry. Quantitation by this method has been found to be reproducible.

10. Analysis of plasma lipoproteins.

(a) Experimental design:

Rabbit progeny from HO and HR parents were caged individually, bled at age ten weeks, fed the cholesterol diet (70 g feed per day containing 200 mg cholesterol) for three weeks and again bled as described above. The plasma lipoproteins from these samples were analysed by preparative ultracentrifugation. Samples of human plasma were obtained by Dr T. G. Redgrave for chemical analysis and by me for immunoelectrophoretic analysis. Two methods of ultracentrifugal preparation of the lipoprotein fractions were used.

(b) Discontinuous density gradient ultracentrifugal method:

A discontinuous density gradient procedure using a high-speed swinging-bucket rotor to separate the plasma lipoproteins into VLDL, LDL and HDL in a single ultracentrifugation was developed in collaboration with Dr T. G. Redgrave who obtained the data on human samples.

Samples of plasma were adjusted to d = 1.21 with solid potassium bromide (0.325 g/ml plasma) and 4.0 ml aliquots were pipetted into 13.5 ml cellulose nitrate centrifuge tubes (9/16 inch diameter x 3½ inch length). Samples of less than 4.0 ml were first adjusted with salt solution d = 1.019 to a volume of 4.0 ml. A

discontinuous gradient was formed by carefully layering 3.0 ml of salt solution d = 1.063 above the plasma, followed by 3.0 ml of salt solution d = 1.019. Finally the tube was filled with 2.5-3.0 ml of d = 1.006 salt solution. To minimise mixing at the density junctions, the salt solutions were allowed to gravity feed down the side of the centrifuge tube through a hypodermic needle (22 gauge) attached to a glass syringe with the barrel removed. All salt solutions contained EDTA (0.1 µg/ml) and were prepared from potassium bromide and sodium chloride (Havel, Eder and Bragdon, 1955).

The samples were centrifuged for 24 hours at 20°C in a rotor (model SW41) in an ultracentrifuge (model L3-50, Beckman Inc., Palo Alto, Calif, 94034, U.S.A.) at 41,000 rev/min (286,000g, R_{max} = 15.23 cm). After centrifugation chylomicrons and VLDL were present at the top of the tube, LDL at the junctions of the next two steps of the density gradient and HDL at the d = 1.21 and d = 1.063 junction. In human samples LDL was clearly visible as an orange-yellow band. The bands, together with part of the salt solutions above and below each band were carefully siphoned into tubes containing ethanol: diethyl ether (3:1 v/v, 40 ml). The tubes were stoppered and warmed in a boiling water bath for approximately ten seconds to denature the protein. After cooling and maintaining at 4°C for two hours the protein was precipitated by centrifugation at 3000g for 20 minutes and the lipid containing supernatant aspirated into flasks. The protein precipitate was washed twice with 20 ml of

diethyl ether and the solvent evaporated from the pooled extract under vacuum on a rotary evaporator. Non-lipid contaminants were removed and neutral lipids separated from phospholipids as described in Section 6 (c,d). The fractions were assayed for the concentration of protein, triglyceride, cholesterol and phospholipid.

(c) Classical ultracentrifugal method:

The method of Havel et al. (1955) was used to separate rabbit plasma lipoproteins into the four density classes obtained by the above method. The same rotor, ultracentrifuge and speed (41,000 rev/min) were used. The plasma was adjusted to d = 1.006 and centrifuged for 10 hours. The top fraction was removed using a tube slicer and the infranate was adjusted to d = 1.019. This was centrifuged for 14 hours, the top fraction removed as before and the infranate adjusted to d = 1.063. This was then centrifuged for 14 hours, the top fraction removed and the infranate adjusted to d = 1.21 and centrifuged for 22 hours after which the top fraction was again removed. All fractions were extracted and assayed as described above.

(d) Immunoelectrophoresis:

An aliquot was removed before extraction and dialysed overnight at 4°C against salt solution d = 1.019 prior to immunoelectrophoresis (Uriel and Grabar, 1956). Electrophoresis in agar using LKB equipment (LKB Produktor A.B., Stockholm 12, Sweden) separated the proteins of the dialysed bands. The purity of the bands was checked with anti-sera to human α_1-lipoprotein, human β-lipoprotein,

whole human serum and to whole rabbit serum purchased from Behringwerke A.G., Marburg-Lahn, Germany. The precipitin lines were stained sequentially for lipid using Oil red O (G. T. Gurr Ltd, London, England, 0.5% w/v) in ethanol (50% v/v) and for protein using light green SF (Allied Chemical and Dye Co, New York, U.S.A., 0.5% w/v) in trichloroacetic acid (5% w/v). With this system protein stains green and lipoprotein brown.

11. Enzyme assay experiments.

(a) Animals:

HO or HR rabbits were killed either before or after three weeks on the cholesterol diet (70 g feed per day containing 200 mg cholesterol). The liver was immediately removed and placed in 0.9% saline at 0-4°C for use in the various enzyme assays.

(b) Hepatic cholesterol synthesis:

A portion of the left lobe of the liver was removed and sliced freehand using a one edged blade. Sodium acetate-1-^{14}C (S.R.A. 0.1 mCi/mM) was added to freshly prepared Krebs-Ringer bicarbonate buffer (0.2 µCi/ml). Liver slices (200 mg) were added to a pre-cooled flask (25 ml) containing buffer (2.0 ml). The flask was gassed with 95% O_2/5% CO_2 for about 10 seconds, firmly stoppered and incubated for 2 hours at 37°C in a reciprocating shaking water bath (Model RW 1812, Paton Industries Pty Ltd, Stepney, S.A., 5069, 200 cycles/min, 65 mm stroke). The reaction was stopped by the addition of 0.2N sulphuric acid (1.0 ml). The contents of the flasks were transferred to saponification tubes with

2x5 ml of water. The samples were then centrifuged at 1000g for ten minutes and the supernatant discarded. Ethanol (2 ml) and 10N KOH (0.5 ml) was added to the precipitate and the mixture heated for 8 hours at 70°C in a water bath. After cooling 1 ml of water was added and the non-saponifiable lipid extracted from the mixture with 2x6 ml petroleum ether (b.p. 60°-80°C). The solvent of the pooled extracts was evaporated under nitrogen and the digitonin-precipitable sterols prepared and washed as described by Sperry and Webb (1950). The sterol digitonides were dissolved in methanol (1.0 ml), transferred to a counting vial with 2x5.0 ml of toluene scintillator and assayed for radioactivity. Calibration curves were used to correct for variable quenching (Appendix 1).

(c) Hepatic cholesterol ester hydrolase:

For use in the first experiment, 1,2-^{3}H cholesteryl oleate was prepared from oleic acid and cholesterol (1,2-^{3}H) by Dr T. G. Redgrave using the method of Swell and Treadwell (1962). Subsequent experiments used (4-^{14}C) cholesteryl oleate (S.R.A. 0.052 mCi/1µM). The assay system was essentially that described by Goodman (1969). A representative sample of liver (10 g) was homogenised with 40 ml of 0.1M potassium phosphate buffer (pH 7.4) using a teflon-glass Potter-Elvehjem homogeniser. The homogenate was centrifuged at 12,000g for 20 minutes to remove cell debris and mitochondria and the supernatant centrifuged for 75 minutes at 105,000g. The centrifuges and rotors used are described in Section 7 (b). The supernatant, excluding fat at the top of the tube (S_{105}),

was used as the source of the enzyme. The labelled cholesteryl oleate was dissolved in acetone and 50 µl (0.01 µCi ^{14}C or 0.03 µCi ^{3}H) added to 2.0 ml of the S_{105} contained in a flask (50 ml). The inhibitor, N-ethylmaleimide (10 µmoles) was added to the control flasks to determine the rate of non-enzymic ester hydrolysis. The mixtures were incubated for 30 minutes at 37°C in a reciprocating shaking water bath (150 cycles/min). The reaction was stopped by the addition of chloroform:methanol (2:1 v/v, 40 ml). The protein precipitate was removed by filtration and the lipid containing filtrate was dried under nitrogen, carrier cholesterol and cholesteryl oleate added and the cholesterol separated from its ester by TLC as described in Section 6(c). The bands of gel containing the cholesterol and cholesteryl ester were scraped into counting vials, toluene scintillator (10.0 ml) added and the contents assayed for radioactivity. Calibration curves were used to correct for variable quenching (Appendix 1).

(d) Hepatic 7α-hydroxylase:

Various methods were used for assaying this enzyme, all with an equal lack of success in the rabbit. There are many reports of the assay of 7α-hydroxylase in rat liver with each group of workers using their own methodology. The various published methods used in the rat are summarised in Table 1. As can be seen the only things common to all methods are the pH and temperature of incubation. One of the problems of the assay is the formation of non-enzymic autoxidation derivatives of cholesterol during the incubation. These include

Table 1. Published methods for the assay of 7α-Hydroxylase.

HOMOGENISING MEDIUM	FRACTION ASSAYED[a]	INCUBATION MEDIUM	CHOLESTEROL MEDIUM	INCUBATION CONDITIONS	AUTHORS
Modified Bucher[b] pH 7.4	S_{20} M_{100} + S_{100}	Modified Bucher[b] pH 7.4	acetone	37°, 60 min	Daniellson et al (1967)
0.25M sucrose	M_{105}	0.1M phosphate buffer pH7.4 Nicotinamide $MgCl_2$, mercapto-ethanol, NADPH generator[c]	Tween 80	37°, 60 min	Gielen et al (1968)
0.25M sucrose Nicotinamide EDTA	M_{105}	0.1M phosphate buffer pH7.4 $MgCl_2$ NADPH generator[c]	Cutscum[d]	37°, 30 min	Shefer et al (1968)
0.25M sucrose	M_{105}	0.1M phosphate buffer, NADPH generator[c] ß-mercapto-ethylamine	acetone	37°, 60 min	Scholan et al (1968)
0.25M sucrose	S_{18}	0.1M phosphate buffer pH7.4 NADPH ß-mercapto-ethylamine	acetone	37°, 60 min	Mitton et al (1971)
	WHOLE CELLS	Sucrose phosphate buffer pH7.4 NADPH generator[c] Nicotinamide EDTA	Tween 20	37°, 20 min	Maver et al (1972)
0.1M phosphate buffer pH 7.4 nicotinamide EDTA	S_{15} M_{104}	0.1M phosphate buffer pH7.4 nicotinamide $MgCl_2$ as for S_{15} + NADPH generator[c]	acetone or Tween 80	37°, 60 min	Mitropoulos et al (1972)
0.25M sucrose ß-mercapto-ethylamine	S_{18}	0.1M phosphate buffer pH7.4 ß-mercapto-ethylamine	acetone	37°, 30 min	Arinoer et al (1973)

a. S refers to supernatant and M to microsomal fractions. The number $x10^3$ refers to centrifugal force (g) i.e. S_{20} is the 20,000g supernatant.

b. Bergström and Gloor (1955).

c. The generating system was NADP, Glucose-6-phosphate and Glucose-6-phosphate dehydrogenase.

d. Isooctyl, phenoxypolyoxethylene ethanol.

7α-hydroxy-, 7β-hydroxy-, 7-keto- and the dihydroxy derivatives of cholesterol. Various autoxidation "inhibitors" have been used, both at the stage of homogenising the liver and during incubation. These are nicotinamide, EDTA and β-mercaptoethylamine (Table 1). In all cases the assumption is made that the radioactive cholesterol is completely miscible with the endogenous cholesterol. However, Mitropoulos and Balasubramaniam (1972) suggest that this may not be correct and that the assay may be influenced by the size of the metabolically active cholesterol pool in the microsomal fraction. The conversion of radioactive cholesterol to 7α-hydroxy-cholesterol is usually 1-5% with the exception of a report by Scholan and Boyd (1968) in which they obtained an 11% conversion.

In the experiments reported here the methods of Mitton, Scholan and Boyd (1971) and Mitropoulos and Balasubramaniam (1972) were used to assay liver microsomal 7α-hydroxylase. Cholesterol 4-^{14}C (S.R.A. 80.54 mCi/mM) and cholesterol 1,2-^{3}H (S.R.A. 31 Ci/mM) were used in the experiments. The reference standards 7α-hydroxy-cholesterol and 7β-hydroxy-cholesterol were prepared by Mr S. D. Turley from cholesterol benzoate via 7-bromo-cholesterol benzoate and 7-formoxy-cholesterol benzoate (Keverling Buisman, Stevens and Vliet, 1947; Henbest and Jones, 1948). Representative samples of liver (10 g) were homogenised in 40 ml of either 0.25M sucrose or 0.1M phosphate buffer (pH 7.4) containing nicotinamide (30 mM) and EDTA (1 mM) with 4 strokes of a Potter-Elvehjem teflon pestle. The

homogenate was centrifuged at 800g for ten minutes to remove cell debris and the resulting supernatant was centrifuged either at 15,000g or 18,000g for 15 minutes to remove mitochondria. The resulting 15,000g (S_{15}) or 18,000g (S_{18}) supernatant was used as the source of the enzyme. The incubation mixture for S_{18} contained an aliquot of S_{18}, β-mercaptoethylamine (10 mM), and 0.1M potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. The incubation mixture for S_{15} contained an aliquot of S_{15} and 0.1M potassium phosphate buffer (pH 7.4) containing nicotinamide (30 mM) and $MgCl_2$ (5 mM) in a final volume of 2.0 ml. Radioactive cholesterol (0.02 µCi ^{14}C or 0.68 µCi ^{3}H) was added in acetone (10 µl) or as a suspension in Tween 80 (1 mg of Tween 80 per incubation, 10 µl).

The mixtures were incubated at 37°C in a shaking water bath (150 cycles/min) for 30 or 60 minutes. The reaction was stopped by the addition of chloroform:methanol (2:1 v/v, 40 ml) and the precipitated protein removed by centrifugation. The lipid-containing supernatant was washed by the method of Folch et al. (1957) and the solvent evaporated under nitrogen. The cholesterol and 7α-hydroxycholesterol contained in the residue were separated by chromatography on thin layers (0.5 mm) of silica gel G on glass plates (50x20 cm) with ether as eluting solvent. The bands and reference standards were visualised under u.v. light with Ultraphor (0.01% w/v in water). The bands corresponding to 7α-hydroxy-, 7β-hydroxy-, 7-keto-cholesterol and cholesterol were scraped into counting

vials. Toluene scintillator (10.0 ml) was added and the samples assayed for radioactivity. Correction curves were used for variable quenching of the tritiated samples (Appendix 1). There was no appreciable quenching for ^{14}C samples.

(e) Plasma lecithin-cholesterol-acyl transferase:

The method of Glomset (1969) was used for this assay. The labelled substrate was prepared as described by Glomset (1969) using 4-^{14}C cholesterol (S.R.A. 80.54 mCi/mM) and heat inactivated rabbit plasma. The enzyme source was fresh plasma obtained from HO and HR rabbits bled either before or after 3 weeks on the cholesterol containing diet (200 mg cholesterol/day). Substrate (1.8 ml, 0.080 µCi) and fresh rabbit plasma (0.2 ml) were mixed and incubated for 30 minutes in air at 37°C in a shaking water bath (150 cycles/min). In some cases 0.2 ml aliquots were removed for analysis at 10, 20, 60 and 180 minutes. The reaction was stopped by the addition of 2 volumes of chloroform: methanol (1:1 v/v) and the protein precipitate removed by centrifugation. Cholesterol was separated from its ester by TLC on silica gel G as described in Section 6(c). The cholesterol and cholesteryl ester bands were scraped into counting vials, toluene scintillator (10.0 ml) added and the samples assayed for radioactivity.

RESULTS AND DISCUSSION

PART I. VARIATIONS IN CHOLESTEROL METABOLISM

1. Effect of piperazine on cholesterol metabolism in rabbits.

Previous experiments, reported by Redgrave and West (1972) had shown that piperazine reduced cholesterolaemia in male cholesterol-fed rabbits but increased cholesterolaemia in female cholesterol-fed rabbits. To investigate the mechanism of this differential effect these studies were repeated using castrated rabbits. Because it has been reported that cytochrome P-450 participates in cholesterol biosynthesis and catabolism (Wada et al., 1968, 1969; Trülzch et al., 1973), the effects of piperazine and castration on the cytochrome P-450 content of hepatic microsomes were studied.

(a) Results:

Throughout the experiments all animals continued to gain weight. Figure 2 shows the pattern of response of plasma cholesterol concentrations when piperazine is added to the diets of male and female castrated rabbits. Piperazine feeding lowers the cholesterol-induced hypercholesterolaemia of male castrates but augments hypercholesterolaemia in females. These results can be compared with the previously published observations of Redgrave and West (1972) using intact rabbits. It is clear that this differential effect of piperazine is observed whether or not the animals have been castrated.

Details of the plasma cholesterol concentrations, total liver cholesterol and liver weights at the end of these experiments are summarised in Table 2. The results

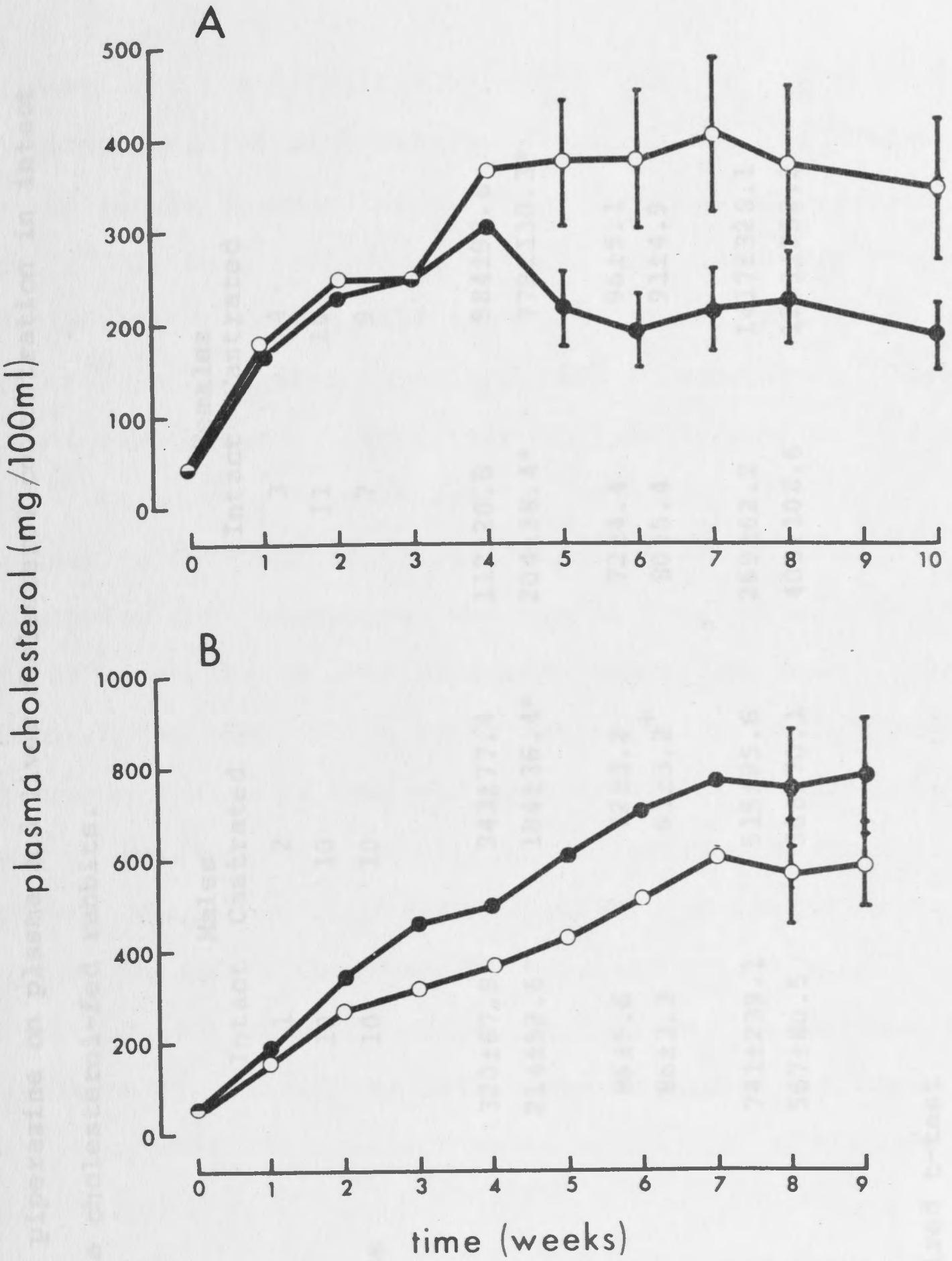


Figure 2. *Effect of piperazine on hypercholesterolaemia in castrated male and castrated female rabbits.* The results of two experiments, (A with castrated male rabbits and B with castrated female rabbits) in which piperazine was added to the diet of cholesterol-fed rabbits: ●—● represents the treated groups, ○—○ control groups not given piperazine. The bars represent 1 S.E. above and below the mean. There were 10 animals in each group in each experiment. In the male rabbits, after a period of 5 weeks, hypercholesterolaemia was reduced in the animals treated with piperazine. On the other hand, in the female rabbits, piperazine has an immediate effect of augmenting hypercholesterolaemia.

TABLE 2. Effects of dietary piperazine on plasma and liver cholesterol concentration in intact and castrated male and female cholesterol-fed rabbits.

	Males		Females	
	Intact	Castrated	Intact	Castrated
Experiment No.	1	2	3	4
Number of rabbits per group	10	10	11	10
Duration of experiment, weeks	10	10	7	9
Plasma cholesterol concentration, mg/dl				
Control	320±67.9	343±77.4	112±20.5	584±93.8
Treated	214±52.6*	184±36.4*	204±38.4*	779±130.3*
Liver weight, g				
Control	86±5.6	72±3.2	72±4.4	96±5.1
Treated	86±3.3	61±3.2+	80±5.4	91±4.9
Liver cholesterol, mg				
Control	741±239.1	515±95.6	269±62.2	1417±328.1
Treated	567±80.5	368±70.1	403±108.6	1251±252.2

Results are mean ± S.E.

* $P<0.05$, comparison by paired t-test

\+ $P<0.05$, comparison by Student's t-test

from experiments with intact rabbits carried out as controls for these studies with castrated animals are also shown. In the intact animals the previous findings of Redgrave and West (1972) were reproduced, except that the liver weights of piperazine-treated male rabbits were not reduced as before. In castrated males however, a reduction of liver weight was observed. It is also apparent from Table 2 that the liver weights of male rabbits were decreased by castration ($P<0.001$ for piperazine-treated groups, $P<0.05$ for control groups) whereas in females an opposite effect was observed ($P<0.01$ for control group only). As shown in the table there is an increase in plasma cholesterol in female castrates compared with controls. However it must be pointed out that pairing of animals was only between control and piperazine-treated groups and not between intact and castrated groups, so whilst a real effect of castration on plasma cholesterol concentration cannot be discounted the difference is within the range of variation which is observed in unpaired groups of animals (see below).

Piperazine, fed without added cholesterol had no effect on the plasma cholesterol concentration of female rabbits (Table 3). After eight weeks on the piperazine diet the mean plasma cholesterol concentration was not significantly different from that of the controls fed plain food with no added piperazine. Redgrave and West (1972) reported that the return of the plasma cholesterol concentration to the pre-cholesterol feeding concentration was unaffected by piperazine in male rabbits.

TABLE 3. Effect of piperazine feeding on plasma cholesterol concentration in female rabbits.

	Mean plasma cholesterol concentration, mg/dl			
Weeks on diet	0	2	4	8
Piperazine-fed	92±3.9	51±5.8	57±6.0	46±2.5
Controls	91±3.6	51±6.0	57±7.0	51±6.0

Results are mean ± S.E., 6 animals per group

The P-450 concentrations of hepatic microsomes of the rabbits used in these studies are shown in Table 4. In all groups except the non-piperazine-treated castrates, female rabbits have higher P-450 concentrations than males. In both intact and castrated males but not females, piperazine treatment lowers P-450 concentrations, whereas in females but not in males, intact rabbits have higher concentrations than castrates for both the control and the piperazine-treated groups. However, due to the differences in liver weight, the differences in the concentration of cytochrome P-450 expressed per weight of microsomal protein are not always mirrored by the changes in total hepatic cytochrome P-450 content (Table 4).

A spectral change occurs when cytochrome P-450 interacts with various substrates (Mitani and Horie, 1969). According to the type of substrate, the difference spectrum is classified as type I (peak 385 nm, trough 420 nm) or type II (peak 420 nm, trough 392 nm). Type I difference

TABLE 4. Hepatic microsomal cytochrome P-450 in intact and castrated cholesterol-fed rabbits and the effects of piperazine treatment.[a]

	Intact Rabbits		Castrated Rabbits	
	Male	Female	Male	Female
1. Concentration, μmoles/g microsomal protein				
Control	1.26±.072	1.89±.114	1.38±.102	1.44±.074
Treated	1.01±.063	1.94±.135	0.94±.096	1.49±.078
2. Total, μmoles/liver				
Control	20.7±1.77	21.5±2.71	15.3±2.03	24.4±2.24
Treated	22.5±2.49	21.8±2.15	8.3±1.85	22.7±1.93

Results are mean ± S.E.

[a] See Table 2 for number of rabbits per group and duration of experiment.

Statistical evaluation, comparisons by Student's t-test:

1. Concentration

   a. In all groups except control castrates, females are higher than males, $P<0.005$
   b. In both intact and castrated males but not females treated groups are less than controls, $P<0.05$
   c. In females but not in males, intact groups are higher than castrates for both control ($P<0.005$) and piperazine treated ($P<0.05$) groups

2. Total

   a. In all castrated groups, females are higher than males, $P<0.01$
   b. In castrated males, treated groups are less than controls, $P<0.02$
   c. In treated males, castrated groups are less than controls, $P<0.001$

spectra were produced with hexobarbital (1 mM) and carbon tetrachloride (1% v/v dispersion). Ethanol (17 mM) produced a type II spectrum. Cholesterol (0.1-0.2 mM) did not interact with cytochrome P-450. Piperazine (0.5-3.0 mM) did not produce a spectral change with cytochrome P-450 nor did it alter the hexobarbital difference spectrum when added prior to the hexobarbital.

Piperazine and cholesterol feeding produced a similar effect in female guinea pigs to that seen in female rabbits, i.e. increased cholesterolaemia. However, male cholesterol-fed guinea pigs, unlike male rabbits, do not show a reduced cholesterolaemia when fed piperazine (S. D. Turley, personal communication). The cytochrome P-450 concentrations of hepatic microsomes from such an experiment are shown in Table 5. There was no significant difference in either the total quantity or the concentration of cytochrome P-450 in the liver for either males or females.

TABLE 5. Hepatic microsomal cytochrome P-450 in cholesterol-fed guinea pigs and the effects of piperazine treatment.[a]

	Male (4)		Female (5)	
	Control	Treated	Control	Treated
Liver wt, g	45±1.4	38±1.2**	39±2.6	35±1.3
Cytochrome P-450, µmoles/g microsomal protein	1.3±0.16	1.5±0.14	1.5±0.11	1.4±0.03
Cytochrome P-450, µmoles/liver	0.4±0.08	0.4±0.06	0.4±0.03	0.3±0.01

Results are mean ± S.E.

[a]duration of experiment, 8 weeks

**significantly different from treated female liver weight. $P<0.01$

(b) Discussion:

To investigate the mechanism of the differential effect of treatment with piperazine on dietary-induced hypercholesterolaemia the logical next step was to repeat the experiments of Redgrave and West (1972) using castrated animals. If the differential effect had been expressed through an effect on sex-hormone control of cholesterol metabolism it would be anticipated that in castrated animals the effect would disappear. However the present experiments show that the effect of piperazine is qualitatively identical in castrated and intact animals. Thus the responses cannot be expressed through a gonad-derived hormone-mediated effect, but must be a manifestation either of a chromosomal sex difference or of a persistent somatic difference conditioned by exposure to sex hormones prior to castration.

The hepatic microsomal cytochrome P-450 system has been shown to be involved in various aspects of hepatic cholesterol metabolism in the rat, both in cholesterol synthesis and in cholesterol catabolism to bile acids (Wada et al., 1968, 1969; Trülzsch et al., 1973). In these experiments the cytochrome P-450 system of rabbits has been examined, to investigate again the possibility of correlations with the changes in cholesterol metabolism produced by piperazine. However no consistent pattern has been found. In piperazine-treated male rabbits a reduction of their hypercholesterolaemia is associated with reduced cytochrome P-450 but the enhancement of cholesterolaemia seen in piperazine-treated female rabbits is not accompanied by an increase of cytochrome P-450.

Similarly cytochrome P-450 was unaffected by piperazine in guinea pigs. These findings support the recent observations of Salvador, Haber, Atkins, Gommi and Welch (1970), who found no clear relationship between changes in serum cholesterol concentrations and the enhancement of hepatic microsomal P-450 induced in rats by treatment with phenobarbital, Clofibrate (ethyl-α[p-chlorophenoxy] isobutyrate) and two other drugs.

The lack of association between cytochrome P-450 content and cholesterolaemia is further illustrated by the observation that castrated female rabbits had a lower cytochrome P-450 concentration but increased cholesterolaemia compared with intact females. However, there is no difference between the total content of cytochrome P-450 per liver in intact and castrated female rabbits. Atkin et al. (1972) have reported that cholesterol synthesis and catabolism in rats is not affected by treatments which change P-450 concentrations. The observation, that microsomal P-450 from rabbits used in these experiments did not produce a spectral change with cholesterol whereas difference spectra were produced by drugs, perhaps lends support to the contention of these authors that there might be different P-450 systems for cholesterol and drugs. Alternatively it is possible that different classes of compound bind at different sites on the one P-450 molecule, or that the increased amounts of endogenous cholesterol in our rabbits have saturated the binding sites for cholesterol.

In rats the cytochrome P-450 concentration of males is greater than females (Kato and Takahashi, 1968) and

castration of males lowers the concentration. The opposite sex difference has been observed in rabbits, i.e. females have higher P-450 concentrations than males, and the difference is removed by castration, which lowers the concentration in females. The differences which have been observed in P-450 content do not always correlate with the changes in liver weight previously noted (Table 2). In some cases total hepatic P-450 content tends to be compensated by the changes in liver weight, but in other cases the difference observed would be exacerbated (Table 4).

The implications of the finding of higher P-450 concentrations in female rabbits than in male rabbits and its contradiction of similar findings in rats are obscure. An understanding of any regulatory role of sex hormones on P-450 activity (or vice versa) is clearly impossible at the present time. Piperazine consistently reduced P-450 content in male rabbits, but the design of the experiments did not allow any conclusions about possible effects of cholesterol feeding on P-450 activity.

2. Variation in plasma cholesterol concentration in non cholesterol-fed rabbits from the randomly outbred population.

Variations in plasma cholesterol concentration between strains and within strains of rabbits have been reported (Laird et al., 1970; Wang et al., 1953). In this study the plasma cholesterol concentrations of large numbers of rabbits in the randomly outbred colony were measured to determine the variation present and to elucidate the causes of such variation.

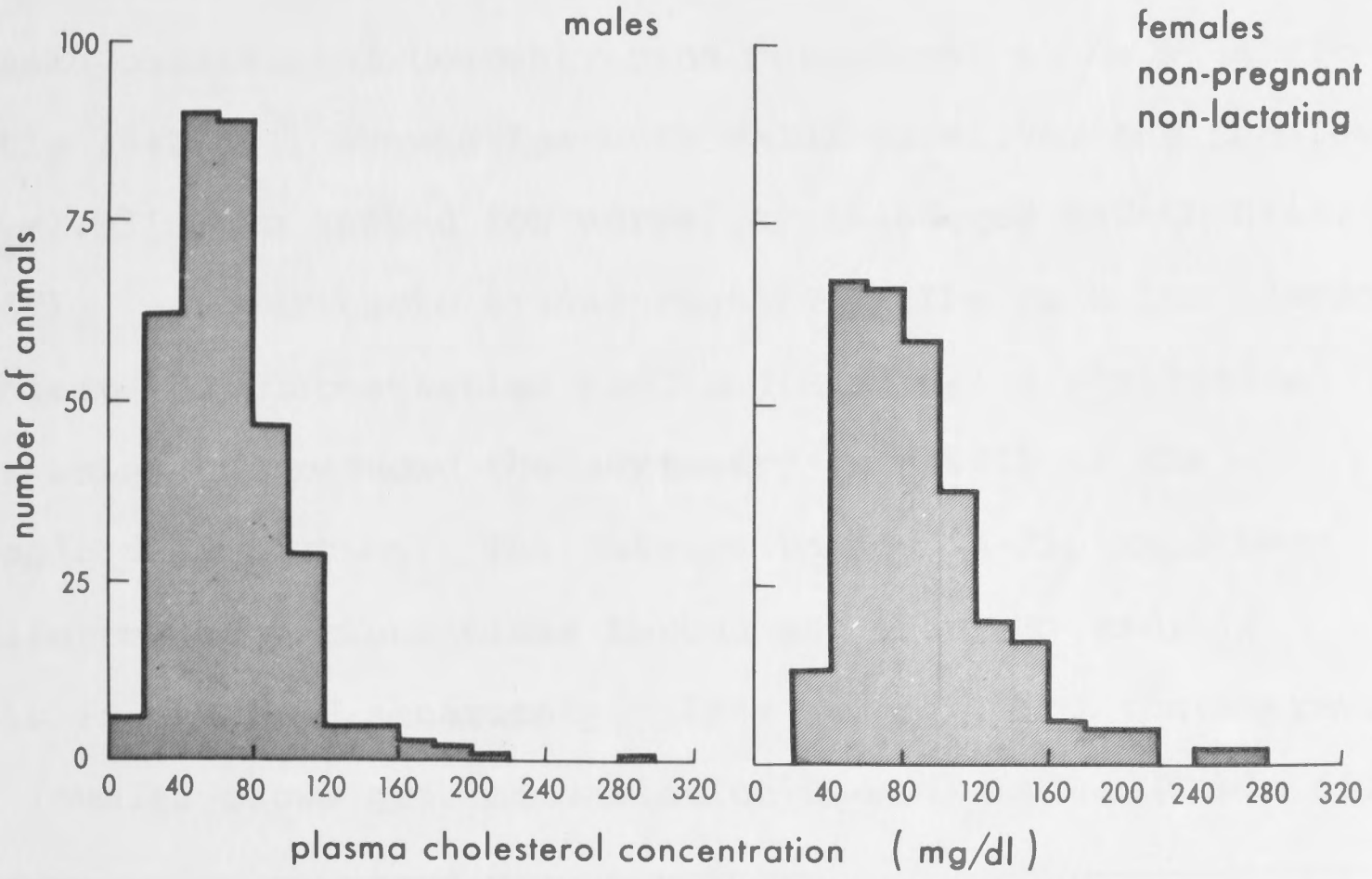


Figure 3. *Frequency distribution of plasma cholesterol concentration of male and non-pregnant, non-lactating female rabbits.*

(a) Results:

(i) Variation between sexes:

Non-pregnant, non-lactating females have a higher mean plasma cholesterol concentration (91±2.6 mg/dl,n=300) than males (68±1.8, n=340). Cholesterol concentration in females ranged from 30-276 mg/dl, and in males from 13-287 mg/dl (Figure 3). The frequency distributions of plasma cholesterol concentration were found to be significantly ($P<0.001$) skewed for both males ($g_1=1.76$) and females ($g_1=1.91$) when tested for normality (Snedecor and Cochran, 1967). A logarithmic transformation of the data for plasma cholesterol concentration produced a normal distribution for males and reduced the asymmetry ($g_1=0.41$) of the female distribution. The data contained in Figure 3 were collected at various times throughout the year and, if this is examined separately, plasma cholesterol concentration in females shows greater variation than in males (Table 6).

TABLE 6. Mean initial plasma cholesterol concentration for male and female rabbits[a]

		Mean ± S.E. mg/dl (n)	
Year	Month	Male	Female[b]
1971	August	63±5.4 (64)	98±5.7*** (71)
	October	64±2.6 (66)	91±6.1*** (42)
1972	January	64±4.7 (59)	58±2.8 (42)
	March	65±8.9 (26)	74±5.6 (43)
	May	79±4.4 (34)	108±8.7** (26)
	August	83±4.9 (21)	103±9.2 (11)
	October	79±4.6 (44)	104±6.4** (35)
	December	56±5.4 (26)	106±8.6*** (30)

a rabbits from randomly outbred population

b non-pregnant, non-lactating

Comparisons by Student's t-test with males of same month

** $P<0.01$ *** $P<0.001$

This observation together with the unequal numbers estimated at the various times throughout the year, may explain the significant ($P<0.01$) skewness still present in the female distribution after logarithmic transformation of the data.

Significantly lower ($P<0.001$) plasma cholesterol concentrations were found in pregnant (37±5.2, n=22) and lactating (52±3.6, n=34) females which were bled at the same time as the non-pregnant, non-lactating females of August 1971 (98±5.7, n=71). Similar lowered plasma cholesterol concentrations in pregnant and lactating females have been reported by Zilversmit, Hughes and Remington (1972). The concentration for lactating females was higher but not significantly different from that for pregnant females and probably represents a stage in the return to the non-pregnant, non-lactating state.

(ii) Variation with age:

Analysis of the variation of initial plasma cholesterol concentration with age did not show a significant correlation for female rabbits ($r=0.046$, n=170). However for the male rabbits between eight and nineteen weeks of age there is a significant ($P<0.001$) negative correlation with initial plasma cholesterol concentration ($r=0.733$, n=350). A semi-logarithmic plot of the regression of mean initial plasma cholesterol on age for male rabbits is presented in Figure 4. Analysis of variance of the components of the regression indicated that deviations from linearity were not significant ($F_{10,238}=2.85$) and so the straight line equation is the line of best fit.

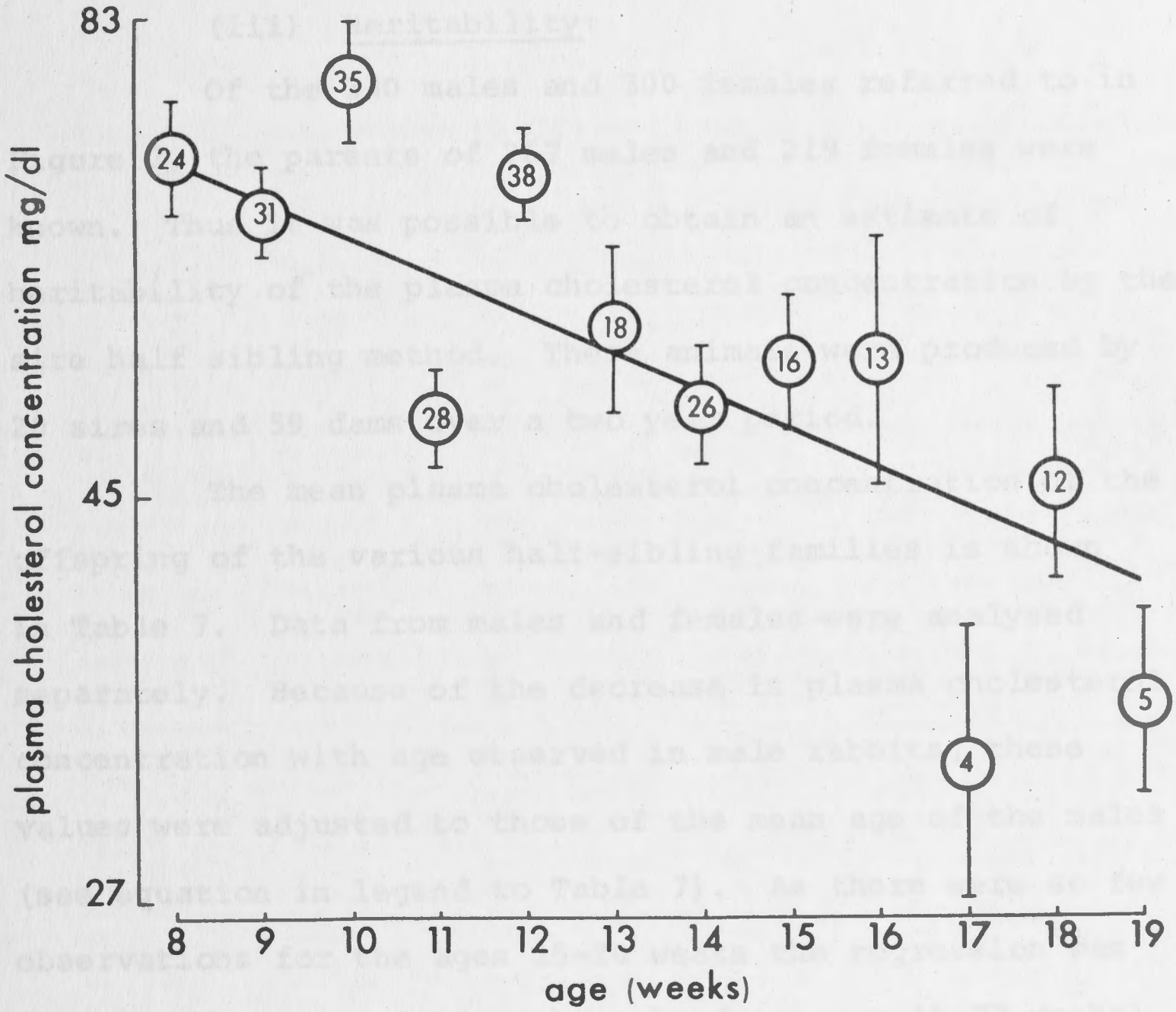


Figure 4. *Regression of the mean plasma cholesterol concentration of non-cholesterol fed male rabbits on age.* The regression is weighted according to the number of observations at each point. The circles, enclosing the number of observations, represent the mean plasma cholesterol concentration with the error bars showing the standard error of the mean. The equation of the line is

$$y = 7.46e^{-0.0200x}$$

where y = log plasma cholesterol concentration

and x = age in weeks

A total of 250 observations were made.

(iii) Heritability:

Of the 340 males and 300 females referred to in Figure 3, the parents of 267 males and 219 females were known. Thus it was possible to obtain an estimate of heritability of the plasma cholesterol concentration by the sire half sibling method. These animals were produced by 20 sires and 59 dams over a two year period.

The mean plasma cholesterol concentration of the offspring of the various half-sibling families is shown in Table 7. Data from males and females were analysed separately. Because of the decrease in plasma cholesterol concentration with age observed in male rabbits, these values were adjusted to those of the mean age of the males (see equation in legend to Table 7). As there were so few observations for the ages 15-20 weeks the regression was calculated on ages 6-14 weeks only (mean age 10.77 weeks) and data from those animals older than this were excluded from the calculation of male heritability and not reported in Table 7. The components of variance of offspring plasma cholesterol concentration for males and females are shown in Table 8. Variation between litters is significantly greater than within litters for both male ($F_{74,192}=1.60$, $P<0.05$) and female ($F_{72,146}=4.25$, $P<0.001$) offspring indicating that the dam is contributing to the variation. This contribution could be both genetic and environmental. Variation between sires is significantly greater than within sires for females ($F_{17,55}=2.40$, $P<0.01$) but not for males ($F_{18,56}=1.74$, N.S..). Thus, the sire is not having a significant effect on the male offspring plasma cholesterol

Table 7. Mean plasma cholesterol concentration of offspring

Dam (Sire)	Plasma cholesterol concentration* Male†	Female
(Bl 1)		
853	42± 2.8(2)	94±15.3(3)
897	70±25.9(9)††	55± 5.0(12)††
907	-	51± 0.8(3)
920	48± 1.2(4)	-
(Bl 3)		
889	47± 1.0(2)	-
897	74± 5.1(3)	-
907	63±11.6(3)	94±13.5(2)
916	-	91 (1)
925	78± 7.6(3)	90±12.0(2)
931	59± 2.2(5)	105±10.0(2)
945	70 (1)	81 (1)
(Bl 4)		
877	55 (1)	-
894	48 (1)	53± 4.4(4)
934	-	68±20.5(2)
(Bl 5)		
894	-	95±13.0(2)
912	-	44± 4.8(4)
(Br 5)		
894	59± 6.6(5)	-
912	46± 4.1(5)	-
(Br 8)		
873	50±13.7(3)	-
896	69±16.0(2)	-
898	50± 4.0(2)	-
905	44 (1)	-
924	71± 2.6(3)	-
936	34 (1)	-
938	-	88±14.7(3)
954	79± 7.1(5)	127±21.1(3)
968	89± 3.5(2)	91±16.0(2)
(Br 9)		
873	-	50±15.5(2)
938	79 (1)	42± 3.6(6)
956	-	63 (1)
962	70± 9.8(5)	81± 3.0(2)
967	72± 7.1(7)	68± 7.9(3)
977	96± 7.7(3)	119± 7.5(5)
(Br 13)		
878	36± 3.6(2)	-
(Gr 5)		
834	66±13.6(3)	86±14.1(3)
944	65± 2.1(6)	-
954	41± 2.2(2)	-
968	-	217±50.9(4)
971	-	98± 1.5(2)
978	70± 8.7(3)	86±16.3(3)
(Gr 6)		
865	83±17.6(4)	-
882	34± 4.5(6)	70±32.0(2)
887	75± 3.9(4)	-
(Gr 7)		
871	-	104±16.7(5)
903	-	94±21.8(3)
923	67± 3.1(4)	123±13.8(4)
929	106 (1)	55 (1)
932	88±11.5(2)	105±11.1(3)
937	-	70±16.5(2)
940	-	64± 0.5(2)
(Gr 8)		
907	71± 1.0(2)	44± 4.9(3)
922	59± 3.3(4)	82± 9.1(4)
925	98±15.4(3)	-
931	83± 6.0(10)††	-
(Gr 9)		
859	65±25.0(3)	42 (1)
874	71± 4.3(4)	81± 7.0(5)
878	56± 5.7(7)††	89±10.0(4)
886	-	104±10.3(5)
887	74± 6.9(9)††	91± 6.5(3)
913	61± 8.8(4)	67± 2.0(2)
919	-	86± 9.5(2)
928	-	68± 7.5(2)
(Gr 11)		
894	51± 8.6(2)	81± 8.0(3)
963	-	67± 2.5(2)
965	98± 8.2(4)	68±13.0(2)
969	-	75 (1)
(Re 5)		
871	83±18.5(3)	146 (1)
918	67±15.7(6)	98± 9.7(9)††
923	62 (1)	-
929	73± 4.9(3)	86±15.6(2)
939	-	61± 3.7(2)
965	-	82±22.6(3)
(Re 6)		
963	75±10.4(4)	150±37.4(4)
970	-	97± 7.9(3)
981	62 (1)	167 (1)
(Sm 2)		
839	88 (1)	93± 8.5(2)
849	56± 8.1(3)	-
894	-	81±12.8(3)
908	63±45.0(2)	-
912	58± 3.9(10)††	-
(Sm 3)		
897	87±12.1(2)	81±11.4(3)
907	80± 9.8(4)	96±13.0(2)
920	58±12.6(2)	138±66.0(2)
925	62± 2.3(7)	70± 4.0(2)
945	86±13.0(2)	111± 5.0(2)
973	-	88±13.9(3)
(Wh 3)		
859	79±37.0(2)	-
865	32 (1)	-
874	88±13.4(5)	155±21.5(2)
878	54 (1)	-
886	113±15.1(10)††	-
887	-	113±16.1(4)
913	58± 9.9(7)	107± 9.1(8)††
928	66±31.0(2)	-
933	56± 5.4(5)	48± 7.5(3)
(Wh 4)		
871	104 (1)	131± 8.0(3)
912	-	90±10.1(4)
918	74±13.9(2)	105±11.6(6)
932	101 (1)	183±10.1(3)
937	82±20.6(5)	191±22.0(3)
939	124±11.2(6)	98 (1)

*Plasma cholesterol concentration of offspring mean ± S.E. mg/dl (n).

†Due to decrease in plasma cholesterol with age the male values have been adjusted to their mean age (10.77 weeks) using the regression equation

$$\log Y = 2.0807 - 0.02445X$$

where:

Y = plasma cholesterol concentration as mg/dl

X = age in weeks.

††Data from two litters.

Table 8. Components of variance of offspring plasma cholesterol concentration

Source	d.f.	Variance	Mean square	F ratio	Expected mean square[a]
MALES					
Total	266	236330			
Between litters	74	91008	1230	1.60*	
Within litters	192	147322	767		W
Between sires	18	32728	1818	1.74	W + 4.488D + 13.822S
Within sires (between dams)	56	58280	1041		W + 3.234D
FEMALES					
Total	218	392410			
Between litters	72	265639	3689	4.25***	
Within litters	146	126771	868		W
Between sires	17	112476	6616	2.40**	W + 3.505D + 11.9731S
Within sires (between dams)	55	153163	2785		W + 2.829D

[a]Expected mean square required for sib analysis of heritability where :

W = component of variance attributable to individuals within full sib families.

D = component of variance attributable to dams.

S = component of variance attributable to sires.

The coefficients of D and S are calculated by the method of Falconer (1963) for unequal family size.

*P < 0.05 **P < 0.01 ***P < 0.001

concentration. This is reflected in the estimate of the heritability for males and females shown in Table 9. The male heritability is low and has a large standard error with respect to the estimate of heritability. In females the heritability of plasma cholesterol concentration is 62±38.8% (h^2±S.E.).

TABLE 9. Heritability of plasma cholesterol concentration in non cholesterol-fed rabbits.

Components of variance[1]	Male	Female
W	767	868
D	85	678
S	49	282
Heritability		
h^2±S.E.	0.22±0.202	0.62±0.388

[a] See footnote a of Table 8 for explanation of W, D and S

(b) Discussion:

The variability in plasma cholesterol concentration in the non cholesterol-fed rabbit is affected by the sex of the animal and the age in males but not females. The variability also has an inherited component. Fillios and Mann (1956) and Laird et al. (1970) have shown that males have lower plasma cholesterol concentrations than females. The decrease with age in male rabbits has also been

reported (Shope, 1928; Geison and Waisman, 1970). However Laird and Fox (1970) who also studied female rabbits observed a decrease for both sexes. This was not shown in the results reported here. This may be explained by the slight seasonal variation among the female rabbits masking any age trend or it may be absent from this strain of rabbits. Increasing the day length has been shown to cause an increase in plasma cholesterol concentration in turkey hens (Mukherjee, Friars and Summers, 1969).

The inheritance of plasma cholesterol concentration has been reported in chickens, mice, beef cattle and rats (Estep, Fanguy and Ferguson, 1969; Weibust, 1973; Stufflebean and Lasley, 1969; Imai and Matsumura, 1973) and the mode of inheritance has been shown to be additive in man (Schaeffer, Adlersberg and Steinberg, 1958), rats (Imai and Matsumura, 1973), mice (Bruell, 1963; Weibust, 1973) and beef cattle (Stufflebean and Lasley, 1969). Sufficient data is not present in this study to make any conclusions concerning the mode of inheritance of plasma cholesterol concentration in the non cholesterol-fed rabbit. Heritability estimates of 26% in male chickens (Estep et al., 1969), 50% in male and female mice (Weibust, 1973) and 73% in male mice (Eapen, Goswami and Pillai, 1971), and 80% in beef cattle (Stufflebean and Lasley, 1969) have been reported. The heritability in female rabbits is shown to be 62±38.8%. The low value for the male heritability (22±20.2%) is difficult to interpret because of the large standard error. It is possible that this particular trait is not heritable in male rabbits.

Certainly, the analysis of variance shows that there is no significant sire effect on the male offspring (Table 8). However, in view of the large error of estimation, the experimental design may not have been appropriate for the assessment of heritability in male rabbits.

3. Variation in plasma cholesterol concentration in the cholesterol-fed rabbit.

Considerable variation in plasma cholesterol concentration in the cholesterol-fed rabbit has been reported by many authors (Rorschneider, 1925; Thölldte, 1927; Turner and Bidwell, 1937; Carroll, 1971). In this study the cholesterolaemic response of rabbits fed the cholesterol diet (200 mg of cholesterol per day) for three weeks is reported.

(a) Results:

(i) Response to cholesterol feeding:

Data from 70 male and 44 female rabbits on diets containing cholesterol (but no other additives) from the piperazine experiments reported here and by Redgrave and West (1972) were analysed. A significant correlation ($P<0.001$) between the initial plasma cholesterol concentration and the increase observed after three weeks on the cholesterol diet was found for both male ($r=0.4553$) and female ($r=0.6912$) rabbits. This correlation was used in the initial selection of dams and sires for the breeding program to select for HO and HR animals as it was possible to predict, with reasonable precision, the type of response from the initial plasma cholesterol concentration.

(ii) Heritability of cholesterolaemic response:

Table 10 presents the data for all the selected matings in terms of the increase in plasma cholesterol concentration of parents and progeny when fed the daily amount of cholesterol containing diet (70 g of diet containing 200 mg cholesterol) for a period of three weeks. From 6 sires and 23 dams a total of 135 progeny were produced. Analysis of variance for the effect of litter size, sex and parents on the cholesterolaemic response of the progeny showed that the only significant effect was that of the parents (Table 11). To test the reproducibility of the cholesterolaemic response, matings of the same parents were repeated and the offspring tested. The variation between litters was very small and not significant (Table 12). Continued feeding of the cholesterol diet after three weeks maintained the difference between HO and HR progeny (Table 13).

The various estimates of the heritability of the cholesterolaemic response are presented in Tables 14 and 15. The components of variance for the full-sibling and half-sibling estimates and their expected mean squares are contained in Table 11. The full-sibling correlation estimate is particularly unreliable in view of the high phenotypic correlation that there is between members of a family (r=0.91). There will, therefore, be a large non-genetic component present in this estimation, which is partly expressed in the large error of the estimate. The half-sibling estimate also has a large error, probably because of the limited number of sires used in this study,

TABLE 10. Mean cholesterolaemic response of parents and progeny of experimental matings in rabbits.

Dam[a] (Sire)		Progeny: Male		Progeny: Female	
		Increase in plasma cholesterol concentration[b] Mean±S.E. mg/dl (n)			
(B5)	45				
946	29	510±115.2	(4)	528	(1)
949	153	419±55.1	(3)	634±33.0	(2)
953	419	148	(1)	203	(1)
957	193	347±68.7	(5)	224	(1)
(G5)	432				
948	236 (2)	849±48.1	(10)	652±9.3	(7)
950	554	625±63.0	(2)	842±129.0	(2)
952	40	572±91.0	(2)	427±83.4	(3)
929	957	300±43.0	(5)	323±45.5	(2)
959	16 (2)	629±191.8	(4)	609±107.2	(9)
(W6)	955				
951	302	1240±83.8	(5)	1180	(1)
953	253 (3)	1053±101.8	(11)	806±148.5	(7)
(G10)	657				
953	419 (2)	928±98.1	(4)	1007±109.1	(8)
951	302 (2)	1056±27.6	(7)	1165±121.8	(4)
964	1095	1034	(1)	820±99.8	(5)
966	993	1405±57.0	(2)	832±54.5	(2)
(S3)	67				
980	200	1059±55.3	(4)	925±226.0	(2)
(B12)	148				
966	993	1074±48.5	(4)	1176±65.1	(5)

[a] Numbers in parentheses refer to number of litters when greater than 1

[b] Increase observed after three weeks on a daily diet of feed containing 200mg of cholesterol

TABLE 11. Components of variance of progeny cholesterolaemic response.

Source	df	Variance	Mean square	F ratio	Expected mean square[a]
Total	134	17,713,378			
Between litters	22	11,683,845	531,084	9.87***	
Within litters	112	6,029,533	53,835		W
Within sexes	133	17,644,713	132,667		
Between sexes	1	68,664		1.93	
Between litter size	6	5,532,689	922,155	2.40	
Within litter size	16	6,151,156	384,447		
Between sires	5	8,590,961	1,718,192	9.44***	W+6.64D+20.90S
Within sires (between dams)	17	3,092,884	181,934		W+5.61D

[a] See footnote a of Table 8 for explanation of W, D and S

*** $P<0.001$

TABLE 12. Mean cholesterolaemic response of full sibling progeny from first and second pregnancies.

Parents		Increase in plasma cholesterol concentration of siblings[a] Mean±S.E. mg/dl (n)	
Sire	Dam	First Pregnancy	Second Pregnancy[b]
G5	948	749±114.6 (7)	749±69.6 (10)
W6	953	1103±100.3 (6)	977±107.7 (9)
G10	951	1153±74.6 (6)	1028±39.4 (5)
G10	953	1177±64.2 (6)	1336±67.1 (6)

[a] See footnote a of Table 10

[b] Comparisons by Student's t-test between siblings in first and second pregnancy are not significant

TABLE 13. Effect of continued feeding of cholesterol diet on cholesterolaemic response of HO and HR rabbits.

Weeks[a]	Increase in plasma cholesterol concentration from pre-cholesterol feeding concentration, mean±S.E. mg/dl	
	HO[b]	HR[b]
3	534±81.7	991±75.7**
4	428±45.0	1251±76.3***
5	528±32.6	1359±79.9***
6	563±69.1	1400±84.0***

[a] No of weeks on cholesterol containing diet (200 mg of cholesterol per day) from age 10 weeks

[b] HO and HR refer to hypo-responder and hyper-responder progeny from breeding program, 5 animals per group

** P<0.01 *** P<0.001

TABLE 14. Heritability of the cholesterolaemic response by regression of progeny on parents.

	Equation[a]	Heritability	n
Regression of progeny:			
on sire	$h^2=2b$	0.49±0.076	7.50[b]
on dam	$h^2=2b$	0.95±0.081	20.02[b]
on mid-parent	$h^2=b$	0.50±0.047	20.66[b]

[a] Definition of mathematical symbols

h^2 = heritability b = regression co-efficient

[b] Weighted total according to the formula $w_n=\frac{n+nT}{1+nT}$

where w_n = weight factor

n = number of offspring and

$T = \frac{t-\beta^2}{1-t}$ for single parent and

$\frac{t-\frac{1}{2}\beta^2}{1-t}$ for mid-parent

where t = phenotypic correlation between members of family and

β = regression co-efficient obtained by calculating unweighted regression of mean progeny response on appropriate parental response

TABLE 15. Heritability of the cholesterolaemic response by correlation of progeny.

Components of variance[a]		
	W	53,835
	D	22,829
	S	72,401
Heritability h^2 ±S.E.		
	Full sibling	1.28±0.261
	Sire half-sibling	1.94±0.749

[a] See footnote a of Table 8 for explanation of W, D and S

there being only six sire half-sibling families. Similar criticisms can also be made of the estimate from the regression of progeny response on sire response. Maternal effects, such as the suckling of the young (see below), contribute to the estimate from the regression on the dam response and could account for the high value (h^2=0.95). However, the regression of progeny response on mid-parent response (Figure 5) will reduce this effect and product a significant regression ($t=2.36$, $P<0.05$) and an estimate of the heritability with the lowest error (0.50±0.047).

(b) Discussion:

Unlike the non-cholesterol fed rabbit, there is no difference between males and females in the magnitude of the cholesterolaemia produced by cholesterol feeding. However, cholesterolaemia is affected by the genetic characteristics of the animal. Adams et al. (1972) have reported that the New Zealand White and Dutch strains of rabbits differ markedly in their susceptibility to induction of hypercholesterolaemia when fed cholesterol. Hypo-responder and hyper-responder Sprague-Dawley rats have been reported by Saito and Fillios (1964). Hyper-cholesterolaemia in man, squirrel monkeys and rats has been shown to be heritable (Fredrickson and Levy, 1972; Clarkson, Lofland, Bullock and Goodman, 1971; Adel, Deming and Brun, 1969). It is clear that the control of such a complex system as the cholesterolaemic response to dietary cholesterol cannot be by the expression of a single gene.

In man, the pathological elevations in cholesterol

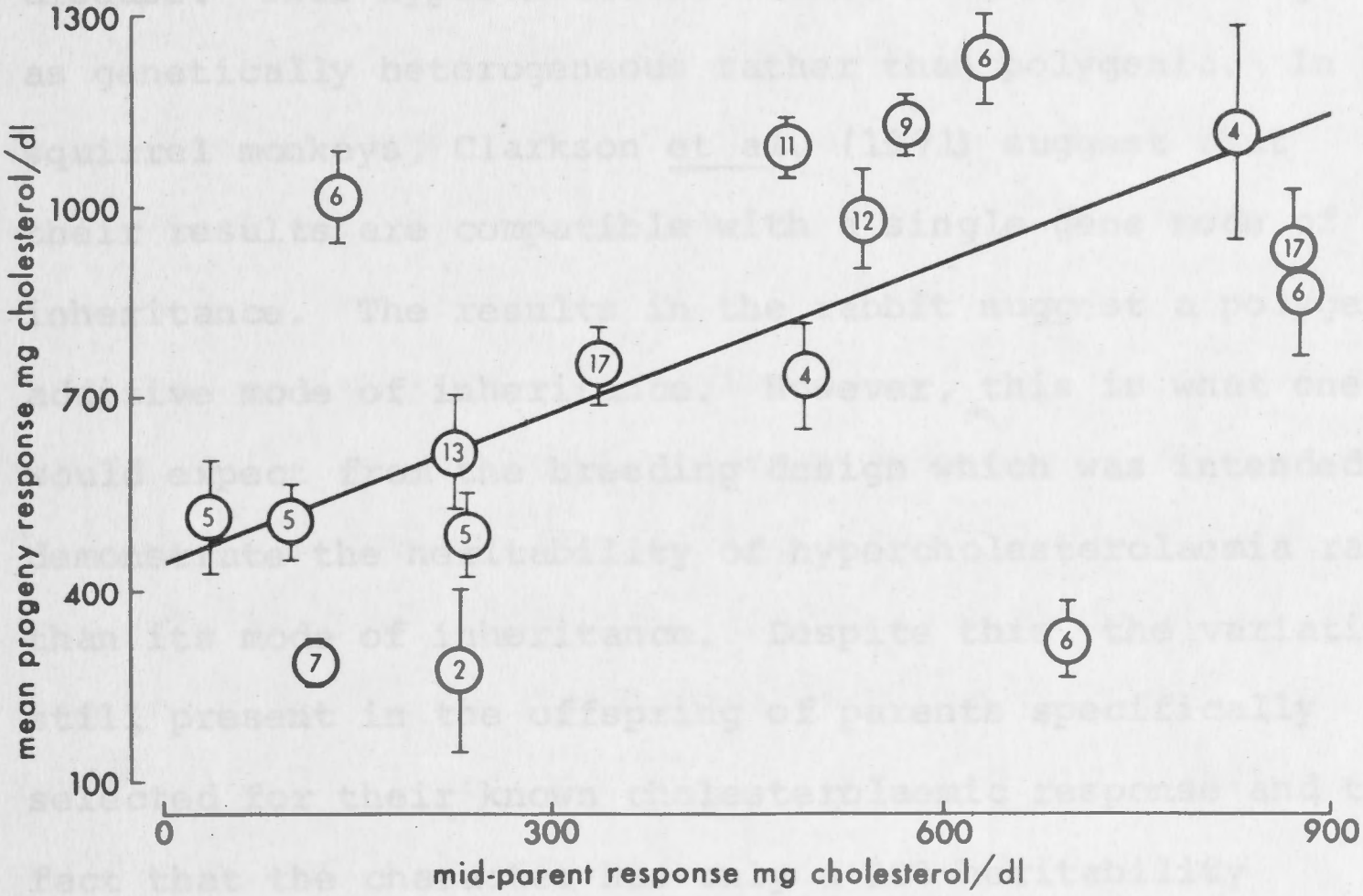


Figure 5. *Regression of mean progeny plasma cholesterolaemic response on mid-parent response.* The cholesterolaemic response was obtained from the increase observed in plasma cholesterol concentration over a three week period on a 0.28% cholesterol containing diet (200 mg of cholesterol/day). A total of 135 progeny from 17 litters was estimated. The circles, enclosing the number of observations, represent the mean cholesterolaemic response with the error bars showing the standard error of the mean.

not only may reflect polygenic factors but also seem to be mediated by several different genes, the best understood being that which causes the syndrome of familial hypercholesterolaemia with xanthomatosis and premature heart disease. Thus hypercholesterolaemia in man can be regarded as genetically heterogeneous rather than polygenic. In squirrel monkeys, Clarkson et al. (1971) suggest that their results are compatible with a single gene mode of inheritance. The results in the rabbit suggest a polygenic additive mode of inheritance. However, this is what one would expect from the breeding design which was intended to demonstrate the heritability of hypercholesterolaemia rather than its mode of inheritance. Despite this, the variation still present in the offspring of parents specifically selected for their known cholesterolaemic response and the fact that the character has only a 50% heritability (50±4.7% calculated from regression of progeny response on mid-parent response) would suggest polygenic inheritance.

It is of interest from a biological viewpoint that the hypo-responding dams were not such good mothers, often failing to raise their young. It was also more difficult to find hypo-responders in the general rabbit colony than hyper-responders. This may be caused by a gene for reduced fertility being associated with one of the genes expressing the hypo-responder character. Reduced fertility has previously been reported in an AX strain of rabbits, autosomal for the gene for buphthalmia, which have a lowered plasma cholesterol concentration compared with normal AX

rabbits (Laird et al., 1970). It has also been reported that hypo-responder rats became infertile after three generations of sibling matings (Imai and Matsumura, 1973).

4. Influence of dam milk on progeny cholesterolaemic response.

With 50% of the cholesterolaemic response being determined environmentally it is possible that the pre-weaning environment determines the response of young rabbits to a cholesterol diet. This is suggested by the increased estimate of the heritability (h^2=0.95) when the regression is determined by progeny response on dam response. To study this possibility litters from HO parents were cross-fostered at birth with litters from HR parents. Feigenbaum and Gaman (1967) have reported that milk from resistant mothers reduces the incidence of spontaneous aortic lesions in non-resistant weanling rabbits.

The effect of milk lipid constituents on the plasma cholesterol concentration of the suckling young have been reported in many species (Shope, 1928; Carroll, 1964; Carroll, Hamilton and Macleod, 1973). The hypercholesterol-aemia seen in the suckling calf and rat has been attributed to the milk triglyceride concentration (Carroll et al., 1973; Harris, MakNintch and Quackenbush, 1966) and in the rabbit to the milk cholesterol (Friedman and Byers, 1961). The effect of various cholesterol intakes in early development on the subsequent cholesterolaemic response to dietary cholesterol has been reported in man, pigs and rats (Glueck, Tsand, Balistreri and Fallat, 1972; Reiser, 1971; Reiser and Sidelman, 1972). In man the plasma cholesterol

concentration at 12 months was uninfluenced by earlier low or moderate cholesterol diets (Glueck et al., 1972). Reiser and Sidelman (1972) report that in male rats after 24 weeks on a cholesterol diet there is an inverse correlation between the dam milk cholesterol concentration and the plasma cholesterol concentration. Reiser (1971) also reports a similar situation for male and female pigs.

Thus, in addition to the cross-fostering experiments, the concentrations of triglyceride, cholesterol phospholipid and protein in milk from HO and HR dams were measured.

(a) Results:

The analysis of milk from four hyper- and four hypo-responder dams are shown in Table 16. The cholesterol, phospholipid, triglyceride and protein concentrations for

---

TABLE 16. Analysis of milk from hypo-responder (HO) and hyper-responder (HR) rabbit dams.

Concentration	Hypo-responder	Hyper-responder
Total cholesterol, mg/dl	46±5.4	93±11.2**
Phospholipid, mg/dl	97±8.1	163±25.3*
Triglyceride, g/dl	13.9±0.98	15.9±1.30
Protein, g/dl	9.2±0.53	8.6±1.54

Results are mean±S.E., four per group

Comparisons are by Student's t-test between hypo- and hyper-responder dams

* $P<0.05$ ** $P<0.01$

hyper-responder dams are similar to those reported by other workers (Bergman and Turner, 1937; Bragdon, 1952). The hypo-responder dams have significantly lower milk total cholesterol ($P<0.05$) but similar protein and triglyceride concentration to the hyper-responder dams. The mean plasma cholesterolaemic response of litters suckled on foster dams together with the response of litters from separate matings of the same parents suckled on their natural mothers are shown in Table 17. It can be seen that the cholesterolaemic response of hyper-responder (HR) offspring remains

---

TABLE 17. Mean plasma cholesterolaemic response of offspring suckled on natural and foster rabbit dams.

Dam	Mean cholesterolaemic response[a], mg/dl±S.E.(n) of offspring suckled on:		
HO[b]	Natural HO	Foster HR	
024	505±73.7 (5)	983±52.0**	(3)
980	-	1036±57.3	(5)
994	512±62.2 (7)	845±71.0**	(5)
HR	Natural HR	Foster HO	
951	1153±74.6 (6)	1072±49.4	(6)
953	1177±64.2 (6)	1096±103.8	(4)
964	856±88.9 (6)	1043±75.5	(6)

[a] Increase observed after three weeks on a daily diet of 70 g of feed containing 200 mg of cholesterol

[b] HO and HR refer to hypo- and hyper-responder rabbits respectively

Comparisons by Student's t-test of offspring cholesterolaemic response between natural and foster mother

** $P<0.01$

the same (HR) whether they are raised on their natural or hypo-responder (HO) foster dams (Table 17). The trait for hyper-response appears to be independent of previous exposure to high or low concentrations in milk of cholesterol and phospholipid. However, HO offspring fostered on HR dams have a significantly higher ($P<0.01$) cholesterolaemic response than HO offspring raised on their natural dams. For HO offspring the increased cholesterol and phospholipid concentrations in the milk of HR dams appears to influence their subsequent cholesterolaemic response so that they resemble HR offspring. Thus the trait for hypo-response would seem to depend for its expression on the milk cholesterol and phospholipid concentrations.

(b) Discussion:

These results differ from those reported by Reiser and Sidelman (1972) in which an inverse relationship between milk cholesterol and the cholesterolaemic response in male rats after 24 weeks on a cholesterol diet was reported. However, an earlier paper by the same authors (Sidelman and Reiser, 1971) reported that female rats raised on high cholesterol formula milk, after 9 weeks on a cholesterol diet, had higher serum cholesterol concentration than those raised on a low cholesterol formula.

Rabbit milk cholesterol is derived principally from plasma cholesterol (Connor and Lin, 1967) and as described above the plasma cholesterol concentration is heritable in non cholesterol-fed female rabbits. The plasma cholesterol concentration at age ten weeks, of the male and female offspring suckled on HO or HR natural or

foster dams did not show any significant differences. However, it was necessary to analyse the male and female data separately because of the sex difference in the plasma cholesterol concentration of non cholesterol-fed rabbits. This means that there were very few observations per litter and consequently the failure to show any significant differences between litters suckled on the various dams may, in part, be due to this. The plasma cholesterol concentration of the four HR dams at ten weeks of age (170±18.4 mg/dl, mean±S.E.) was significantly higher ($P<0.01$) than that of the four HO dams (74±5.3). This could account for the difference in milk cholesterol concentration. The plasma cholesterol concentration of the dams, however, was not measured at the time of milk collection. The increased phospholipid concentration in the milk of HR dams may simply be associated with the increased cholesterol concentration in the milk.

PART II. STUDIES ON HYPO-RESPONDER AND HYPER-RESPONDER RABBITS

As shown in Part I, it has been established that there are two types of cholesterolaemic response to dietary cholesterol and that these traits are heritable. This section describes the investigations undertaken to determine the biochemical differences between hypo-responder and hyper-responder rabbits.

1. Transport of cholesterol in the lipoproteins.

To study the transport of cholesterol in the lipoproteins, plasma was collected from HO and HR rabbits before and after the three week period on the cholesterol diet (200 mg cholesterol per day). The plasma was then separated into four lipoprotein classes using a discontinuous density gradient ultracentrifugal method.

(a) Results:

(i) Validation of ultracentrifugal method:

A single discontinuous gradient ultracentrifugal method was developed, in collaboration with Dr T. G. Redgrave, to study the distribution of cholesterol within the lipoproteins of HO and HR rabbits. The method separated the lipoproteins into VLDL ($d<1.006$) LDL_1, ($1.006<d<1.019$), LDL_2 ($1.019<d<1.063$) and HDL ($1.063<d<1.21$). In order to validate the method, plasma from a hyper-cholesterolaemic rabbit was separated into the above density classes by this method and by the classical method of Havel et al. (1955). The distribution of cholesterol, triglyceride, phospholipid and protein within the lipoprotein

fractions were then compared. In addition, immuno-electrophoresis of the fractions separated by this method in both humans and rabbits was carried out to determine the purity of the fractions when tested against mono-specific antisera to α_1-lipoprotein and β-lipoprotein.

Data on the cholesterol, triglyceride, phospholipid and protein concentrations of the various lipoprotein fractions isolated by the classical method of Havel et al. (1955) and by the discontinuous density gradient method from a hypercholesterolaemic rabbit are presented in Table 18. Recovery of loaded material by the classical method was 98.1±0.81% and by this method was 92.4±2.27%. Both methods produced similar values for the composition of fractions I and II (VLDL and LDL_1). However, fraction III (LDL_2) prepared by the classical method has more lipid and protein than fraction III prepared by this method. These differences are more marked in fraction IV (HDL). Calculations using the relationship described by Dole and Hamlin (1962) for flotation of lipoprotein species show that centrifugation for 24 hours is sufficient for quantitative recovery of all VLDL and LDL_1 ($1.006<d<1.019$) in the correct region of the gradient. However, centrifugation for 41 hours is required for quantitative recovery of LDL_2 ($1.019<d<1.063$). For all of the HDL to migrate to the top of the d=1.21 band, centrifugation for 55 hours is required. As the centrifugation time in the discontinuous density gradient method was only 24 hours, less lipid and protein were recovered in fractions III and IV by this method than by the classical method (Table 18). However,

TABLE 18. Concentrations of the lipoprotein constituents of plasma from a hypercholesterolaemic rabbit

Fraction	I	II	III	IV	V	TOTAL	
Density	<1.006	1.006-1.019	1.019-1.063	1.063-1.21	>1.21	(by addition)	(measured)
Designation	VLDL and chylomicrons	LDL_1	LDL_2	HDL	-		
Triglyceride[a]							
classical[b]	43	23	13	4	1	84	87
1 spin[c]	43	23	7	3	0.5	76.5	
Total cholesterol							
classical	141	117	60	23	1	342	348
1 spin	130	114	60	19	6	329	
Phospholipid							
classical	37	69	41	21	8	176	177
1 spin	44	68	31	15	10	168	
Protein							
classical	32	50	71	79	-	232	-
1 spin	29	42	60	61	-	192	-
Total lipoprotein							
classical	253	259	185	127	-	-	-
1 spin	246	247	158	98	-	-	-

[a] Results are mg/dl.

[b] classical ultracentrifugal method of Havel et al. (1955)

[c] discontinuous gradient method developed in this laboratory.

the percentage distribution of components within a fraction is very similar for both methods (Table 19). The distribution of protein, triglyceride, total cholesterol and phospholipid within VLDL, total LDL (LDL_1 plus LDL_2) and HDL is similar to that reported for cholesterol-fed rabbits by Garlick and Courtice (1962) and Garlick, Courtice and Munoz-Marcus (1965).

TABLE 19. Distribution of the lipoprotein constituents within the lipoprotein species of plasma from a hypercholesterolaemic rabbit

Fraction[a]	Composition %			
	I	II	III	IV
Triglyceride				
Classical	17	9	7	3
1 spin	17	9	4	3
Total cholesterol				
Classical	55	45	33	18
1 spin	53	46	38	19
Phospholipid				
Classical	15	27	22	17
1 spin	18	28	20	16
Protein				
Classical	13	19	38	62
1 spin	12	17	38	62
Total				
Classical	100	100	100	100
1 spin	100	100	100	100

[a] for designation and density of fraction; see Table 18

As separation is dependent on the density of the lipoprotein species, separations using the discontinuous density gradient method should be quite distinct. Immunoelectrophoresis in agar of the fractions isolated by the discontinuous gradient method showed that there was no cross contamination of LDL and HDL and that each fraction was free of other plasma proteins (Table 20). As monospecific antisera to rabbit α_1- and β-lipoproteins were not available, anti-whole rabbit serum was used. With this

TABLE 20. Presence of precipitin lines in rabbit lipoprotein fractions after immunoelectrophoresis.

Fraction[a]	No of precipitin lines to anti-whole rabbit serum
I	1
II	1
III	1
IV	1
Whole serum	many

[a] See Table 18 for densities and designation of fractions

anti-serum, fractions I-IV showed only single lipid staining precipitin lines (Figure 6). However the position and shape of the precipitin lines of fractions III and IV clearly show that they are different lipoproteins.

Immunoelectrophoresis of human lipoproteins prepared by this method also showed that there was no contamination of the LDL fractions with HDL. The HDL fraction was also free of LDL and all fractions were free of other plasma proteins (Table 21). Fraction IV showed

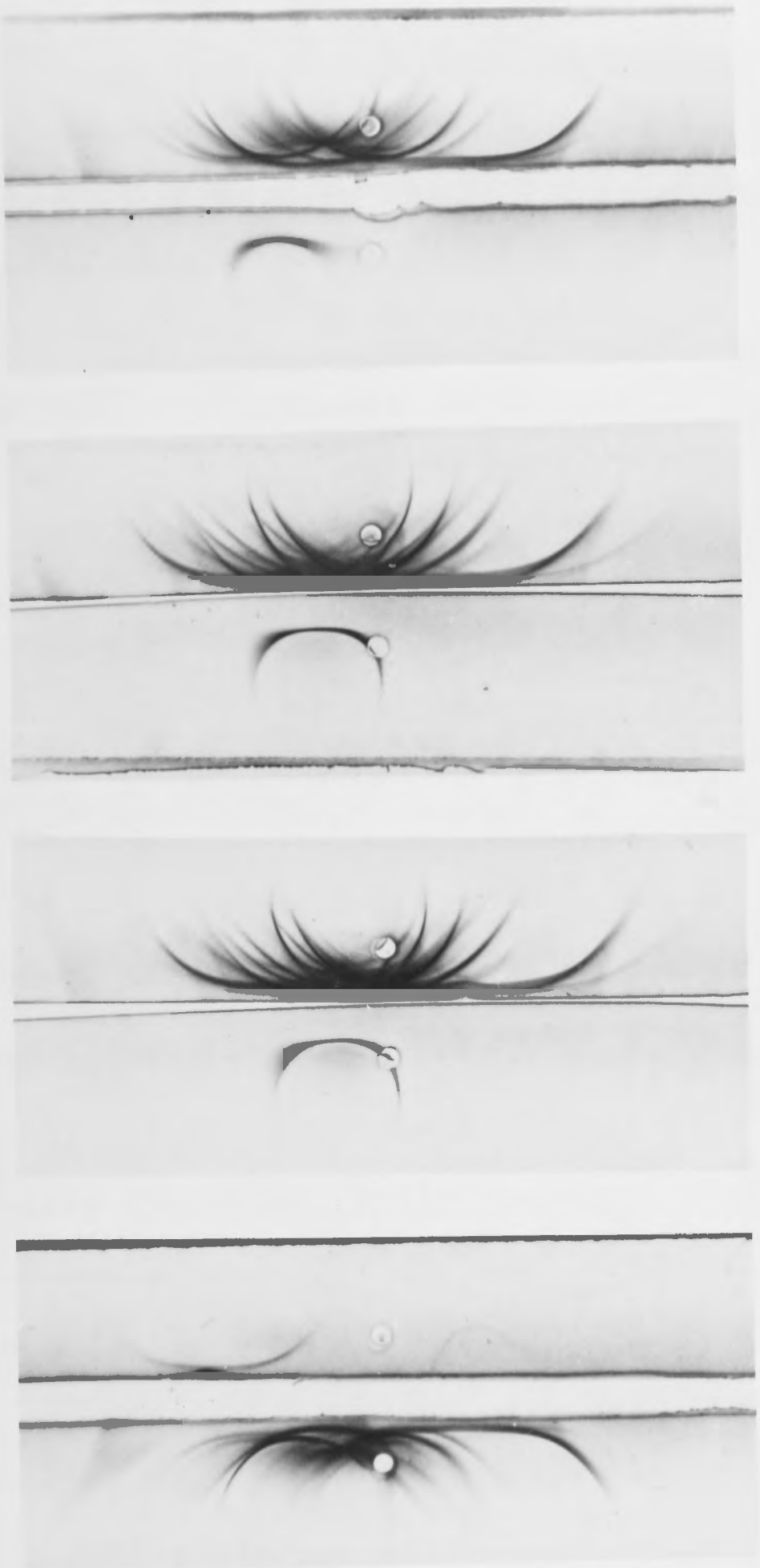


Figure 6. *Immunoelectrophoresis of rabbit lipoproteins isolated by ultracentrifugation.* The fractions were dialysed at 4°C overnight against saline d = 1.019. Electrophoresis was carried out on aliquots of the fractions at 250 volts for 1 hour using LKB apparatus. Veronal (barbital) buffer pH 8.6, ionic strength 0.1 was used.

aH = anti-whole rabbit serum
Hs = whole rabbit plasma
I = fraction I from ultracentrifugation
II = fraction II " "
III = fraction III " "
IV = fraction IV " "

See Table 18 for densities and designations of fractions.

TABLE 21. Presence of precipitin lines to specific anti-sera in human lipoprotein fractions after immuno-electrophoresis.

Fraction[a]	Number of precipitin lines to various anti-sera[b]		
	anti-whole	anti-α_1[c]	anti-β[c]
I	(1)	0	(1)
II	(1)	0	(1)
III	1	0	1
IV	1	1	0
V	many	1	0
Whole serum	many	1	1

[a] See Table 18 for densities and designations of bands

[b] ( ) indicate the presence of a weak line

[c] Anti-sera to α_1- and β-lipoproteins.

---

only a single lipid staining precipitin line with anti-α_1-lipoprotein serum and with anti-human serum. Similarly fraction III showed only a single precipitin line with anti-β-lipoprotein serum and with anti-human serum (Figure 7). Anti-β-lipoprotein serum showed a weak precipitin line with fractions I and II but anti-α_1-lipoprotein did not react. Fraction V ($d<1.21$) showed a single lipid staining precipitin line with anti-α_1-lipoprotein and with anti-human serum (Table 21) indicating the presence of some residual HDL in this fraction. It is also possible that VHDL might be present or that some dissociation of the lipoproteins has occurred during

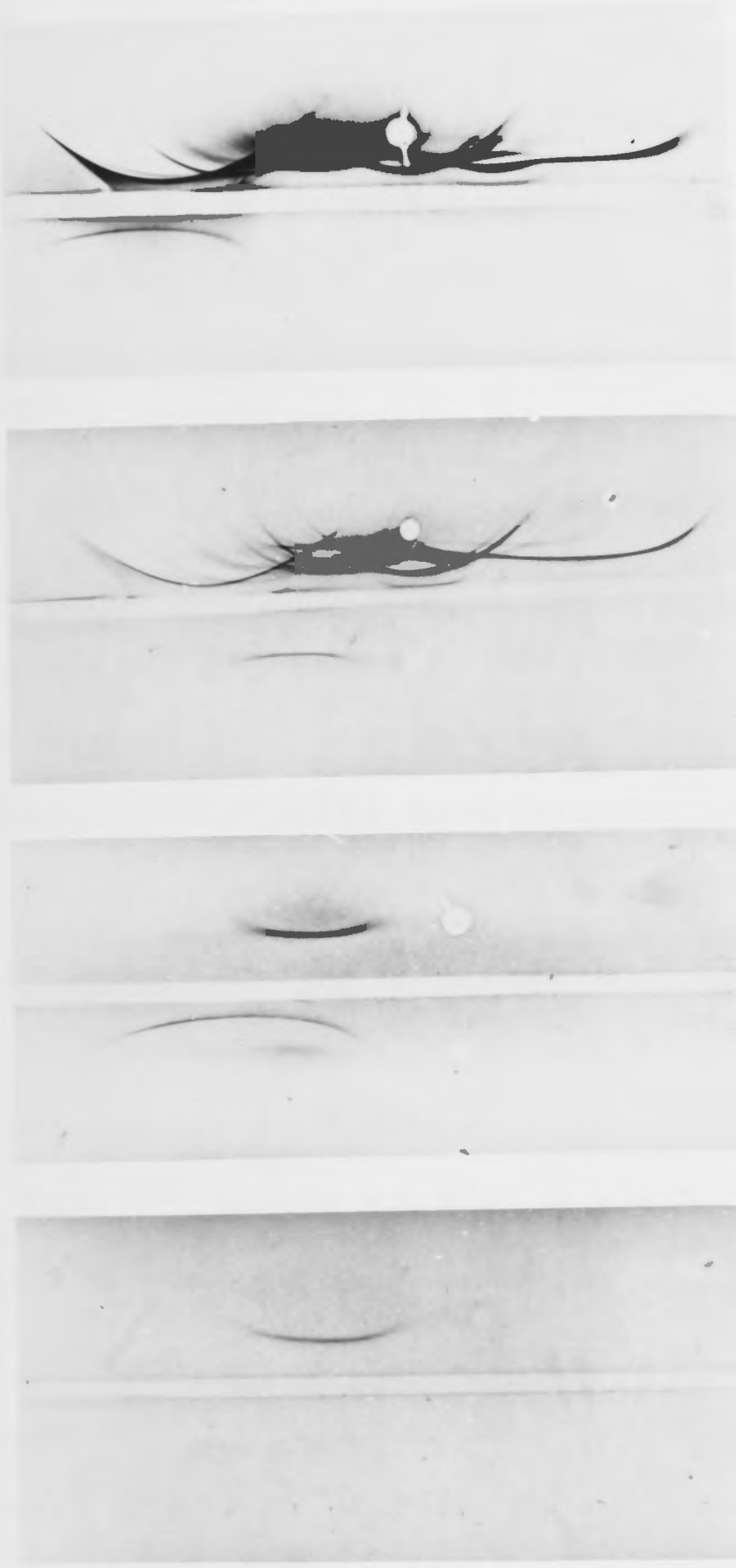


Figure 7. *Immunoelectrophoresis of human lipoproteins isolated by ultracentrifugation.* The fractions were dialysed at 4°C overnight against saline d = 1.019. Electrophoresis was carried out on aliquots of the fractions at 250 volts for 1 hour using LKB apparatus. Veronal (barbital) buffer pH 8.6, ionic strength 0.1 was used.

aH = anti-whole human serum
aB = anti-β-lipoprotein serum
Hs = whole human plasma
III = fraction III from ultracentrifugation
IV = fraction IV " "

See Table 18 for densities and designations of fractions.

ultracentrifugation (Levy and Fredrickson, 1965). With electrophoresis on cellulose acetate, fractions III and IV travel as single bands corresponding to LDL and HDL (T. G. Redgrave, personal communication).

As demonstrated by immunoelectrophoresis and by comparison with the classical method, the discontinuous density gradient method provides adequate quantitative separation of the lipoproteins into VLDL, LDL_1, LDL_2 and HDL. This method does not separate chylomicrons and VLDL as both these lipoproteins have $d<1.006$. As chylomicrons circulate for only a brief period after the ingestion of fat (Harris and Felts, 1970), they should not be present in plasma of rabbits fasted overnight. Thus fraction I will consist entirely of VLDL.

(ii) Analysis of plasma lipoproteins from non cholesterol-fed HO and HR rabbits:

Data on the mean concentration of triglyceride, total cholesterol, phospholipid and protein from six non cholesterol-fed HO and HR rabbits aged ten weeks are shown in Table 22. Recoveries of loaded material were similar for triglyceride (93.5±6.38%), phospholipid (91.8±1.19%) and total cholesterol (91.3±1.34%). There were no differences between non cholesterol-fed HO and HR rabbits in the concentrations of triglyceride, phospholipid, total cholesterol and protein. Cholesterol is carried predominantly in fractions II and III (total LDL) and triglyceride in fraction I and II (VLDL and LDL_1). The percentage composition of the lipoprotein classes are shown in Table 23. Both HO and HR rabbits

TABLE 22. Concentrations of the lipoprotein constituents of plasma from non cholesterol-fed HO and HR rabbits[a]

Fraction[b]	I	II	III	IV
Triglyceride				
HO	18±2.3	18±2.0	10±1.0	6±0.8
HR	13±1.9	14±2.0	11±1.4	8±0.9
Total cholesterol				
HO	7±1.0	35±3.4	25±3.3	14±2.5
HR	8±0.9	26±3.3	21±1.2	17±1.3
Phospholipid				
HO	9±1.1	32±1.7	50±8.6	41±8.3
HR	8±0.8	23±2.8	40±1.9	38±2.4
Protein				
HO	21±2.6	33±5.5	52±8.0	82±5.8
HR	21±3.7	29±3.7	38±2.8	90±5.2

Results are mean±S.E. mg/dl, 6 animals per group

[a] HO and HR refers to hypo-responder and hyper-responder rabbits respectively

[b] See Table 18 for designation and density of fractions.

TABLE 23. Distribution of lipoprotein constituents within the lipoprotein species of plasma from non cholesterol-fed HO and HR rabbits[a]

Fraction[b]	Composition, % I	II	III	IV
Triglyceride				
HO	32±3.3	15±1.3	8±0.7	4±0.7
HR	27±3.2	15±0.9	10±1.1	5±0.5
Total cholesterol				
HO	13±1.4	30±1.9	19±1.4	10±1.8
HR	16±0.9	28±0.7	19±0.9	11±0.7
Phospholipid				
HO	16±1.4	28±1.8	36±4.4	28±4.4
HR	15±0.9	25±0.8	36±0.9	25±1.6
Protein				
HO	39±5.5	27±3.4	37±3.7	58±5.6
HR	42±3.6	32±0.9	35±1.7	59±2.5
Total				
HO	100	100	100	100
HR	100	100	100	100

Results are mean±S.E., 6 animals per group

[a] HO and HR refers to hypo-responder and hyper-responder rabbits respectively

[b] See Table 18 for designation and density of fractions.

have similar distribution of lipid and protein within the lipoprotein classes. These results differ markedly from those reported by Mills and Taylaur (1971) for a normal rabbit. Although the total lipoprotein concentration for HDL and total LDL are similar, the percentage composition is different for all three lipoprotein classes (VLDL, LDL and HDL). However, only one domestic rabbit of unspecified strain was analysed by Mills and Taylaur (1971) and this may account for the discrepancy.

(iii) Analysis of plasma lipoproteins from cholesterol-fed HO and HR rabbits:

The mean distribution and composition of the plasma lipoproteins of non cholesterol-fed and cholesterol-fed HO and HR rabbits are illustrated in Figures 8 and 9. The concentration of protein increases and triglyceride decreases with increasing density. Cholesterol feeding results in a dramatic increase in cholesterol concentration, especially in fraction I (VLDL). Increases in the concentrations of phospholipid and protein in VLDL and LDL_1 (fraction II) are associated with this increase in cholesterol concentration. This is in agreement with the work of Gofman, Lindgren, Elliot, Mantz, Hewitt, Strisower, Herring and Lyon (1950) which showed that after the initial increase in the S_f5-8 component with a density of 1.03 there followed the appearance of several components of S_f10-30 and higher. These components would correspond to LDL_1 and VLDL. Data on the mean concentrations of triglyceride, total cholesterol, phospholipid and protein from the six HO and HR rabbits after three weeks on the

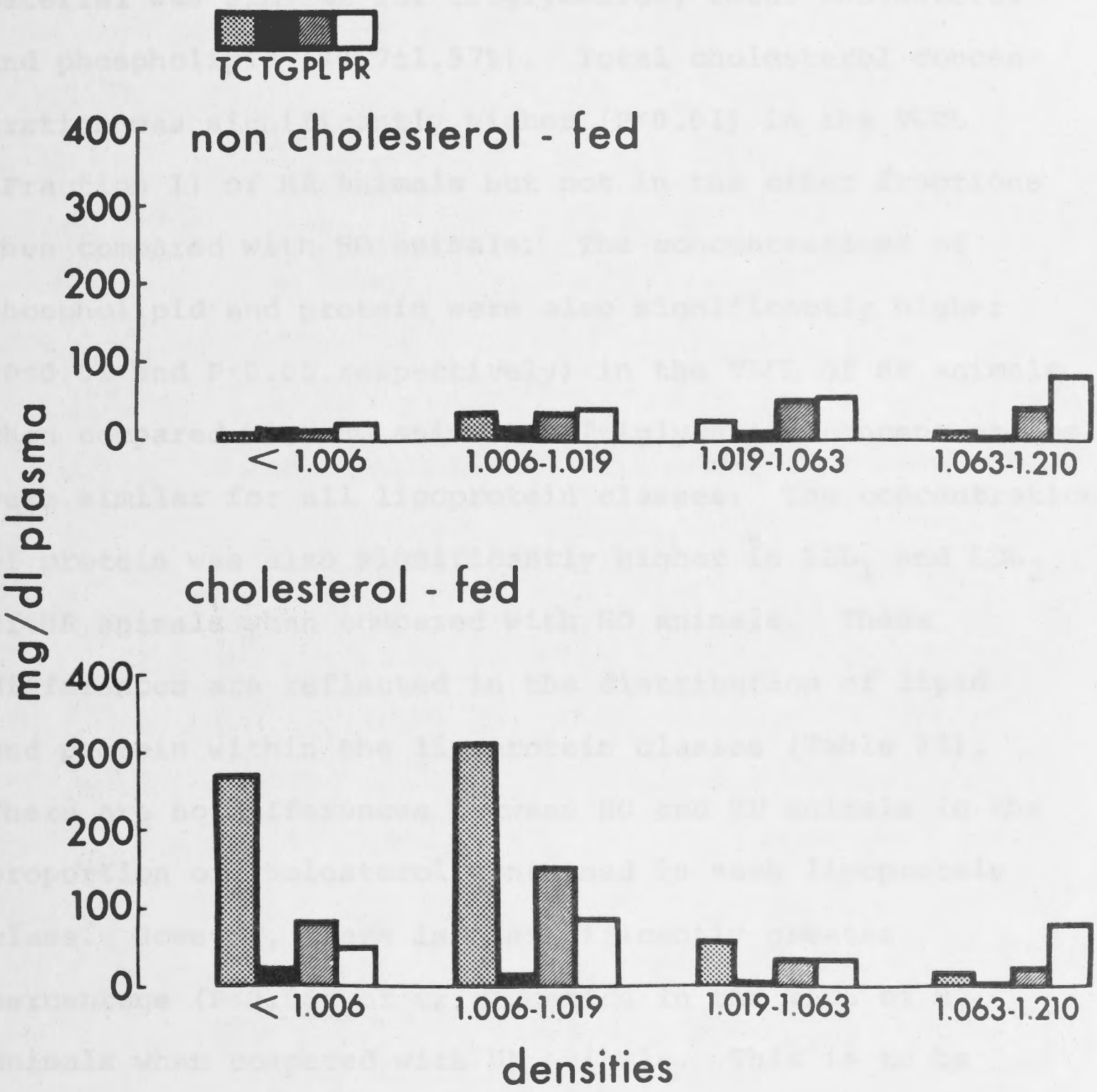


Figure 8. *Concentrations of lipoprotein constituents within density classes of plasma from hypo-responder rabbits.* Mean of six animals

TC = Total cholesterol TG = Triglyceride
PL = Phospholipid PR = Protein

cholesterol diet are shown in Table 24. Recovery of loaded material was similar for triglyceride, total cholesterol and phospholipid (90.7±1.57%). Total cholesterol concentration was significantly higher ($P<0.01$) in the VLDL (Fraction I) of HR animals but not in the other fractions when compared with HO animals. The concentrations of phospholipid and protein were also significantly higher ($P<0.01$ and $P<0.05$ respectively) in the VLDL of HR animals when compared with HO animals. Triglyceride concentrations were similar for all lipoprotein classes. The concentration of protein was also significantly higher in LDL_1 and LDL_2 of HR animals when compared with HO animals. These differences are reflected in the distribution of lipid and protein within the lipoprotein classes (Table 25). There are no differences between HO and HR animals in the proportion of cholesterol contained in each lipoprotein class. However, there is a significantly greater percentage ($P<0.05$) of triglyceride in the VLDL of HO animals when compared with HR animals. This is to be expected from the fact that there was no difference in triglyceride concentration between HO and HR animals but there was decreased concentration of the other three constituents of VLDL in HO animals (Table 24). A recent report by Shore, Shore and Harb (1974) states that the VLDL of cholesterol-fed New Zealand White rabbits contains "4% protein, 13% phospholipid, very little glycerides and large amounts of cholesteryl esters". Their results differ from those reported here in the percentage of phospholipid and protein but agree with regard to triglyceride and cholesterol (Table 25).

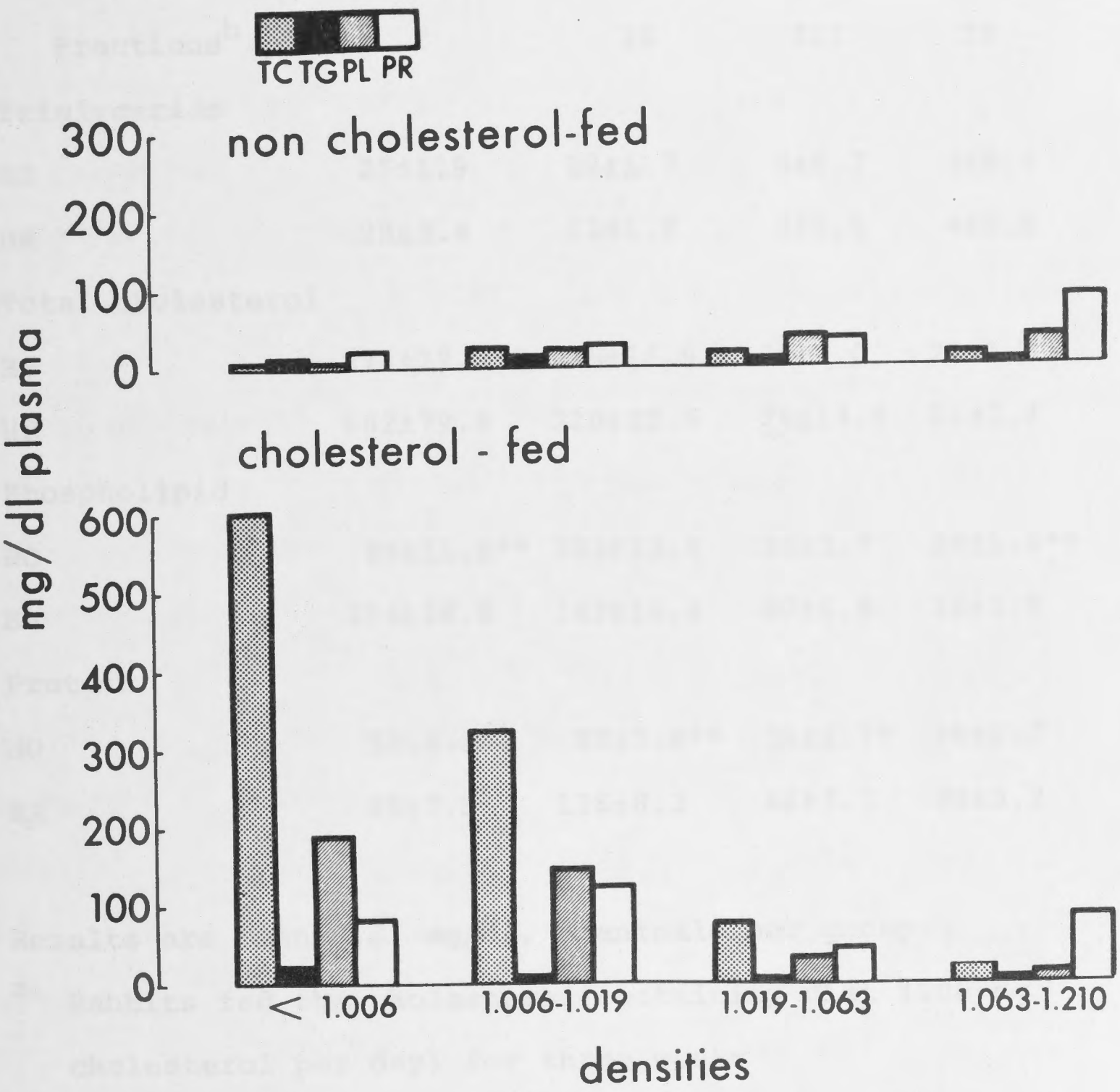


Figure 9. *Concentrations of lipoprotein constituents within density classes of plasma from hyper-responder rabbits.* Mean of six animals

TC = Total cholesterol TG = Triglyceride

PL = Phospholipid PR = Protein

TABLE 24. Concentrations of the lipoprotein constituents of plasma from cholesterol-fed HO and HR rabbits[a]

Fractions[b]	I	II	III	IV
Triglyceride				
HO	25±1.9	12±1.7	4±0.7	3±0.4
HR	25±3.4	12±1.8	4±0.5	4±0.6
Total cholesterol				
HO	274±22.2**	315±16.9	60±7.0	20±1.0
HR	602±79.8	320±32.5	78±14.6	20±2.9
Phospholipid				
HO	89±11.8**	153±13.8	36±3.7	24±1.6**
HR	194±24.6	147±10.4	37±1.8	16±1.9
Protein				
HO	53±8.3*	87±7.4**	36±2.7*	80±9.3
HR	85±7.9	126±8.3	46±3.3	86±5.2

Results are mean±S.E. mg/dl, 6 animals per group

a Rabbits fed the cholesterol containing diet (200 mg cholesterol per day) for three weeks

b See Table 18 for designation and density of fractions.

Comparisons by Student's t-test between HO and HR of same fraction and lipid group.

* $P<0.05$ ** $P<0.01$

TABLE 25. Distribution of lipoprotein constituents within the lipoprotein species of plasma from cholesterol-fed HO and HR rabbits[a]

	Composition, %			
Fractions[b]	I	II	III	IV
Triglyceride				
HO	6±0.8*	2±0.3	3±0.4	2±0.3
HR	3±0.5	2±0.3	2±0.1	3±0.4
Total cholesterol				
HO	62±1.4	56±0.7	44±1.6	16±0.7
HR	66±1.2	52±2.1	46±2.9	15±1.2
Phospholipid				
HO	20±1.1	27±1.2	27±1.5	20±1.4**
HR	21±0.9	25±0.6	23±1.7	13±1.0
Protein				
HO	12±1.8	15±1.5*	26±0.8	62±1.9*
HR	10±0.3	21±1.6	29±2.0	69±2.1
Total				
HO	100	100	100	100
HR	100	100	100	100

[a] HO and HR refer to hypo-responder and hyper-responder rabbits respectively

[b] See Table 18 for designation and density of fractions.

Comparisons by Student's t-test between HO and HR of same fraction and lipid group.

* $P<0.05$ ** $P<0.01$

The distribution of lipid and protein within HDL are similar in HR rabbits to those reported by Garlick and Courtice (1962) who studied cholesterol-fed rabbits. However, HO rabbits have significantly more phospholipid and significantly less protein in HDL than HR rabbits (Table 25).

(b) Discussion:

Cholesterol is transported in the lipoproteins of HO and HR animals in a similar way. The additional plasma cholesterol in HR animals is found solely in VLDL and this is associated with increased protein and phospholipid concentrations. Rose (1972) reported that in rabbits, unlike man, the liver is the major source of the cholesteryl esters of VLDL arising from cholesterol feeding. This is a significant finding as HR rabbits have lower liver cholesteryl ester concentration than HO rabbits. Thus it is possible that the hyper-responder (HR) rabbit is less able to retain cholesterol in the liver than the hypo-responder (HO) rabbit. Camejo, Bosch, Arreaza and Mendez (1973) report that increased concentrations of intracellular cholesterol leads to increased lipoprotein synthesis in the rabbit and this may account for the increased plasma cholesterol concentration seen in the cholesterol-fed HR rabbit.

2. Steroid balance study.

To determine the absorption and synthesis of cholesterol and the excretion of neutral and acidic steroids derived from cholesterol, steroid balance studies were undertaken during steady state conditions for both HO and HR rabbits. The metabolic steady state is assumed to be attained when constant values of plasma cholesterol concentration, body weight and faecal steroid excretion are maintained over a period of a few weeks. Under these conditions input, i.e. synthesis plus dietary intake, is balanced by outflow, i.e. excretion. Thus synthesis equals excretion minus intake. If the daily intake of cholesterol is isotopically labelled during this period, the rate of excretion of radioactivity will equal the rate of intake and the specific radioactivity of the plasma cholesterol will become constant, i.e. an isotopic steady state will be achieved. The combination of the balances of mass and radioactivity provide the information necessary to determine cholesterol absorption and hence entry. The equations used to determine these parameters and their derivation as described by Grundy and Ahrens (1969) are contained in Table 26. In this study eleven HO and ten HR animals were fed 4-^{14}C cholesterol (100.1 mμCi daily) with the diet. For a period of eight weeks the daily intake of cholesterol was 16 mg and in the subsequent five weeks was 217 mg. Faecal collections were made weekly from weeks four to thirteen of the experiment on six HO and six HR rabbits. One of the HO rabbits died during week seven of the experiment and thus the steroid balance was calculated for

TABLE 26. Equations for calculating cholesterol synthesis, absorption and entry from data provided by steroid balance study[a].

1. Daily cholesterol synthesis (mg/day) = daily total faecal steroids [neutral + acidic] corrected for neutral steroid losses and variations in faecal flow (mg/day) - daily cholesterol intake (mg/day).

2. Daily cholesterol absorption = daily intake - daily unabsorbed cholesterol
   where: daily unabsorbed cholesterol = total faecal neutral steroids [specific radioactivity of neutral steroids - specific radioactivity of plasma cholesterol] ÷ [specific radioactivity of dietary cholesterol - specific radioactivity of plasma cholesterol].

3. Daily cholesterol entry[b] = daily cholesterol absorption ÷ fraction of plasma cholesterol derived from absorbed cholesterol.

[a] From Grundy and Ahrens (1969).

[b] The term entry is used in preference to "turnover" used by Grundy and Ahrens (1969). It defines the entry of new cholesterol into miscible body pools.

only five HO animals. The cause of death was unrelated to the experimental procedure.

(a) Results:

(i) Plasma cholesterol concentration, specific activity and body weight throughout the experiment:

The mean plasma cholesterol concentrations for the HO and HR rabbits are shown in Figure 10. It can be seen that there are no differences in plasma cholesterol concentration between HO and HR animals prior to cholesterol feeding. However, after only one week on the cholesterol diet the HR animals have a significantly higher ($P<0.05$) plasma cholesterol concentration (207±11.8 mg/dl) than the HO animals (143±20.0 mg/dl) and this difference is maintained for the five weeks the animals were fed the cholesterol diet. It is interesting to note the increase in plasma cholesterol concentration is considerably less than that observed for animals phenotyped as HO and HR during the breeding program. This may be an age effect as the animals used in these experiments were between 20 and 30 weeks of age whereas all animals were 10 weeks of age at the start of the three week cholesterol feeding period used to phenotype the progeny of the breeding program.

For the first six weeks the mean plasma cholesterol specific activity was higher for HR rabbits (Figure 11). This difference was not significant. However for the last two weeks on the normal diet (16 mg cholesterol/day) and for the first two weeks of cholesterol feeding (217 mg/day) the difference was significant.

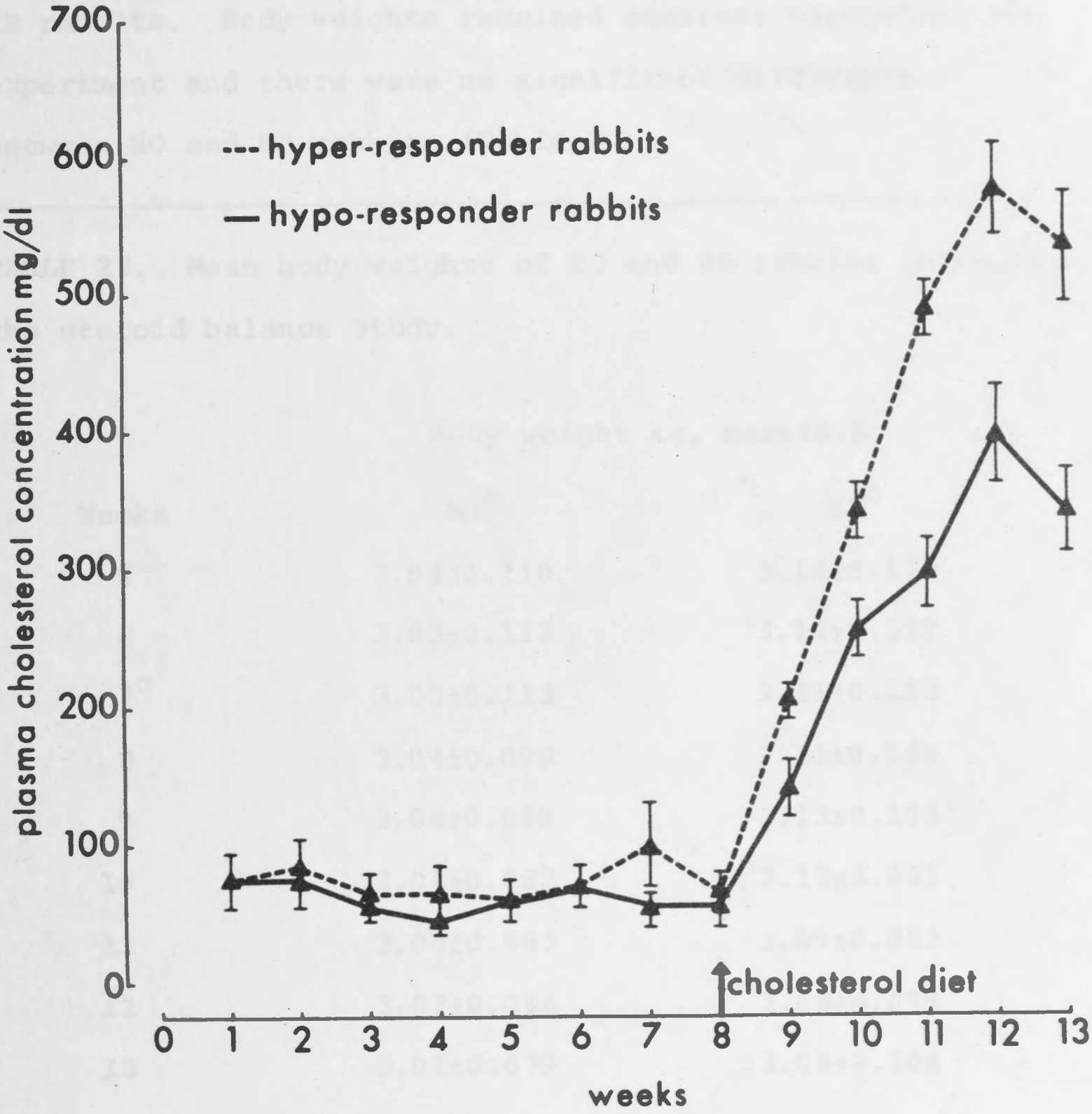


Figure 10. *Plasma cholesterol concentrations of hypo- and hyper-responder rabbits.* The points represent the mean plasma cholesterol concentration with the bars representing one S.E. above and below the mean. There were 11 animals in the hypo-responder group up to week 7 and 10 animals thereafter. There were 10 animals in the hyper-responder group throughout the experiment.

However after this time there was no significant difference in the mean plasma cholesterol specific activity of HO and HR rabbits. Body weights remained constant throughout the experiment and there were no significant differences between HO and HR rabbits (Table 27).

TABLE 27. Mean body weights of HO and HR rabbits throughout the steroid balance study.

Weeks	Body weight kg, mean±S.E. HO[a]	HR[b]
5	3.04±0.110	3.14±0.174
6	3.03±0.112	3.14±0.117
7[c]	3.05±0.113	3.11±0.113
8	3.04±0.098	3.11±0.109
9	3.04±0.089	3.13±0.103
10	3.04±0.087	3.13±0.081
11	3.00±0.083	3.09±0.083
12	3.03±0.084	3.08±0.094
13	3.01±0.079	3.08±0.104

a Eleven hypo-responder animals measured to week 7 and ten animals thereafter

b Ten hyper-responder animals were measured throughout the experiment

c Commencement of cholesterol feeding (200 mg/70 g feed per day).

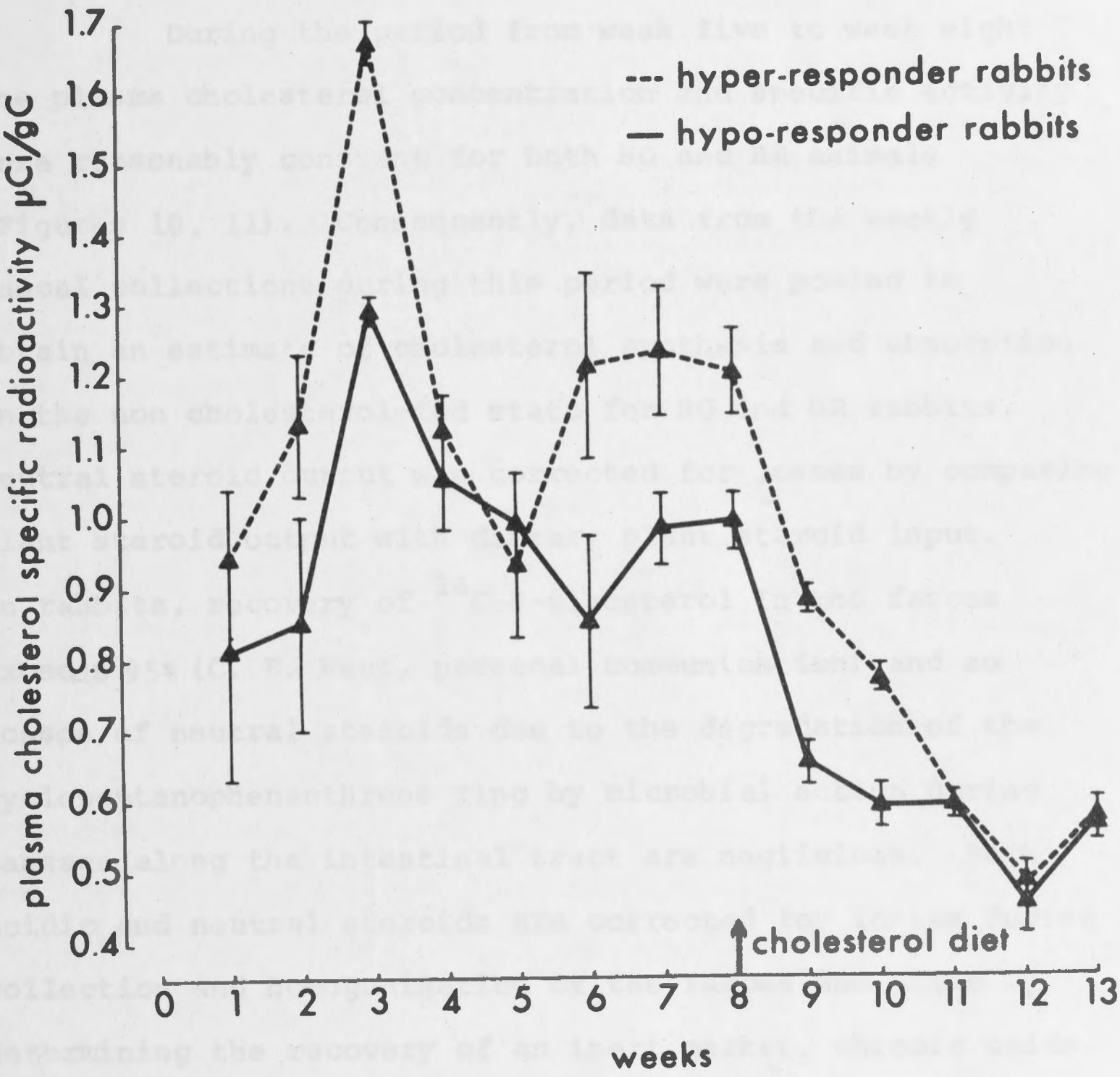


Figure 11. *Plasma cholesterol specific radioactivity of hypo- and hyper-responder rabbits.* The points represent the mean plasma cholesterol specific radioactivity with the bars representing one S.E. above and below the mean. See legend to figure 10 for number of animals per group.

(ii) Steroid balance for HO and HR rabbits on a diet containing no added cholesterol:

During the period from week five to week eight the plasma cholesterol concentration and specific activity were reasonably constant for both HO and HR animals (Figures 10, 11). Consequently, data from the weekly faecal collections during this period were pooled to obtain an estimate of cholesterol synthesis and absorption in the non cholesterol-fed state for HO and HR rabbits. Neutral steroid output was corrected for losses by comparing plant steroid output with dietary plant steroid input. In rabbits, recovery of ^{14}C-β-sitosterol in the faeces exceeds 95% (C. E. West, personal communication) and so losses of neutral steroids due to the degradation of the cyclopentanophenanthrene ring by microbial action during passage along the intestinal tract are negligible. Both acidic and neutral steroids are corrected for losses during collection and homogenisation of the faeces and urine by determining the recovery of an inert marker, chromic oxide. Recoveries of the internal neutral steroid standard, 7α-^{3}H-cholesterol were generally 95% after saponification and extraction and 90% after TLC of the neutral steroids. Recoveries of the acidic steroid standard, either ^{3}H-cholic acid or ^{3}H-deoxycholic acid, were generally from 50-60%. Such low recoveries result from the difficulties of the extraction procedure.

The output of neutral and acidic steroids for the five HO and six HR rabbits is shown in Table 28. Bile acids could not be quantitated individually because their

TABLE 28. Balance of neutral and acidic steroids in HO and HR rabbits - non cholesterol-fed.

		Balance, mg/day		
	Dietary[a]	Faecal steroids		
HO	cholesterol	Neutral	Acidic[b]	Total
1	16	27	52	79
2	16	29	17	46
3	16	35	26	61
4	16	36	30	66
5	16	31	18	49
Mean±S.E.	-	32±1.7	29±6.3	60±6.0
HR				
1	16	16	37	53
2	16	22	40	62
3	16	28	34	62
4	16	32	19	51
5	16	27	23	50
6	16	33	36	69
Mean±S.E.	-	26±2.6	32±3.4	58±3.1

a Two constituents of the diet were meat and fish meal which contain cholesterol

b Bile acid output is expressed in terms of cholesterol. The molecular weight of dihydroxycholic acid was used in these calculations.

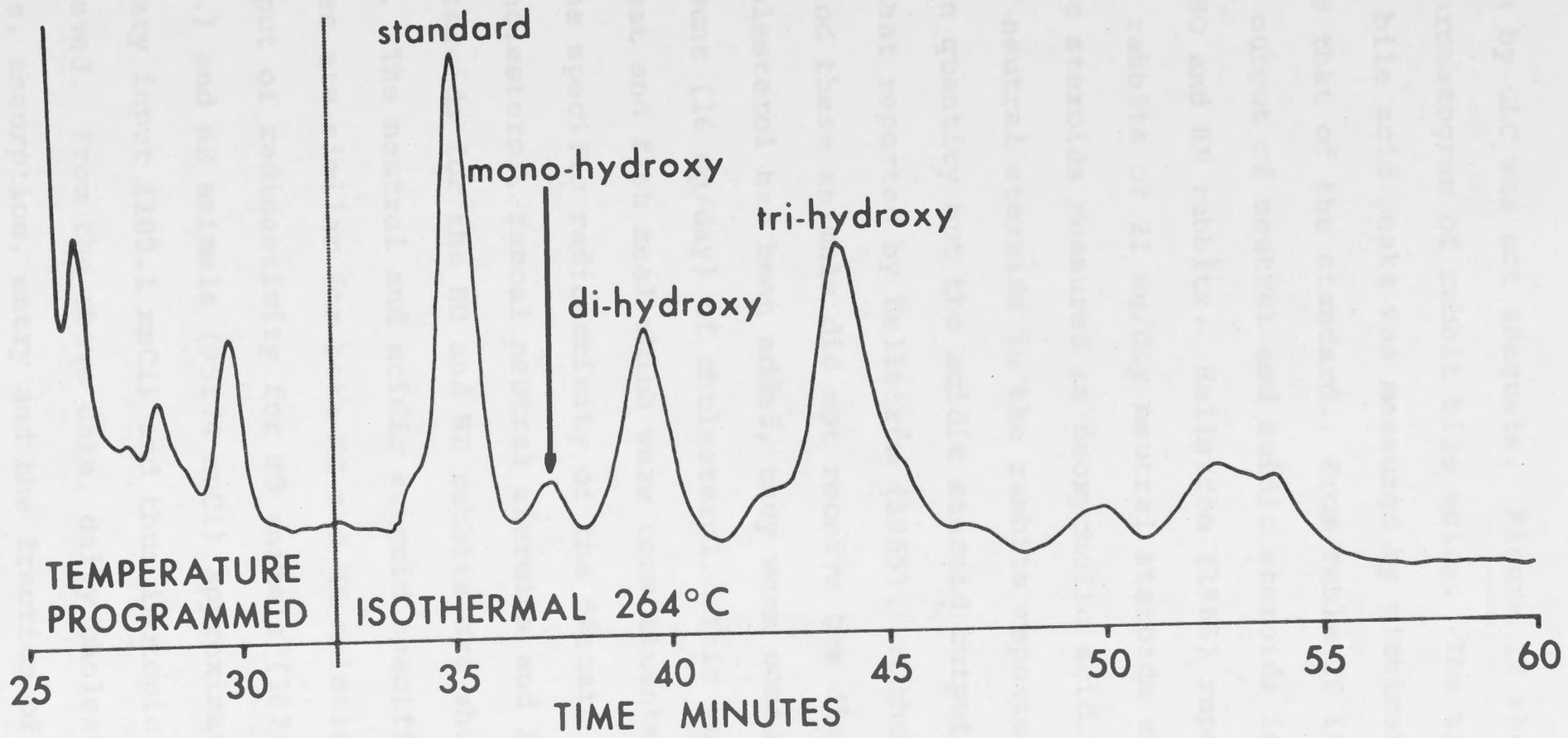


Figure 12. *Chromatogram of the trimethylsilyl derivatives of rabbit acidic steroid methyl esters separated by GLC.* The column was stainless steel (3 metres, 1/8 in O.D.) packed with Gas Chrom Q (100-120 mesh) coated with JXR (3% w/w). The standard is 5β-cholestan-3β-ol. See text for details of gas flow rates and temperature programming.

separation by GLC was not adequate. Figure 12 shows a typical chromatogram of rabbit bile acids. The total area under the bile acid peaks was measured by planimetry and related to that of the standard. From Table 28 it can be seen that output of neutral and acidic steroids is similar for both HO and HR rabbits. Hellström (1965) reports an output in rabbits of 21 mg/day neutral steroids and 68 mg/day acidic steroids measured as deoxycholic acid. The output of neutral steroids in the rabbits reported here is similar in quantity but the acidic steroid output is only half of that reported by Hellström (1965). Although during this period these animals did not receive the diet to which cholesterol had been added, they were consuming a small amount (16 mg/day) of cholesterol. This was present in the meat and fish meal which were constituents of the diet. The specific radioactivity of the dietary cholesterol, plasma cholesterol, faecal neutral steroids and faecal acidic steroids for the HO and HR rabbits are shown in Table 29. The neutral and acidic steroid specific radioactivities are similar for both HO and HR animals. The mean output of radioactivity for HO animals (107±5.7 mμCi, mean±S.E.) and HR animals (95±74 mμCi) approximated the dietary input (100.1 mμCi) and thus isotopic balance was achieved. From the above data, daily cholesterol synthesis, absorption, entry and the fraction of plasma cholesterol derived from absorbed cholesterol were determined using the equations in Table 26. The results of such determinations are presented in Table 30.

TABLE 29. Specific radioactivity of neutral and acidic steroids in HO and HR animals - non cholesterol-fed

		Specific activity of steroids, μCi/gC[a]		
			Faecal steroids	
HO	Dietary cholesterol	Plasma cholesterol	Neutral	Acidic
1	7.47	0.90	1.30	1.29
2	"	1.15	1.66	2.45
3	"	1.01	2.47	1.28
4	"	0.88	1.68	1.41
5	"	1.11	2.40	2.47
Mean±S.E.	-	1.01±0.054	1.90±0.229	1.78±0.279
HR				
1	7.47	1.16	2.11	1.83
2	"	1.01	1.92	1.31
3	"	1.20	1.70	2.11
4	"	1.11	1.48	2.01
5	"	1.01	1.15	1.54
6	"	0.82	1.27	1.75
Mean±S.E.	-	1.05±0.056	1.60±0.154	1.76±0.121

[a] Results are expressed as μCi/g carbon to correct for the molecular weight differences between cholesterol and the bile acids. The molecular weight of dihydroxycholic acid was used in these calculations.

TABLE 30. Parameters of cholesterol metabolism in HO and HR rabbits - non cholesterol-fed

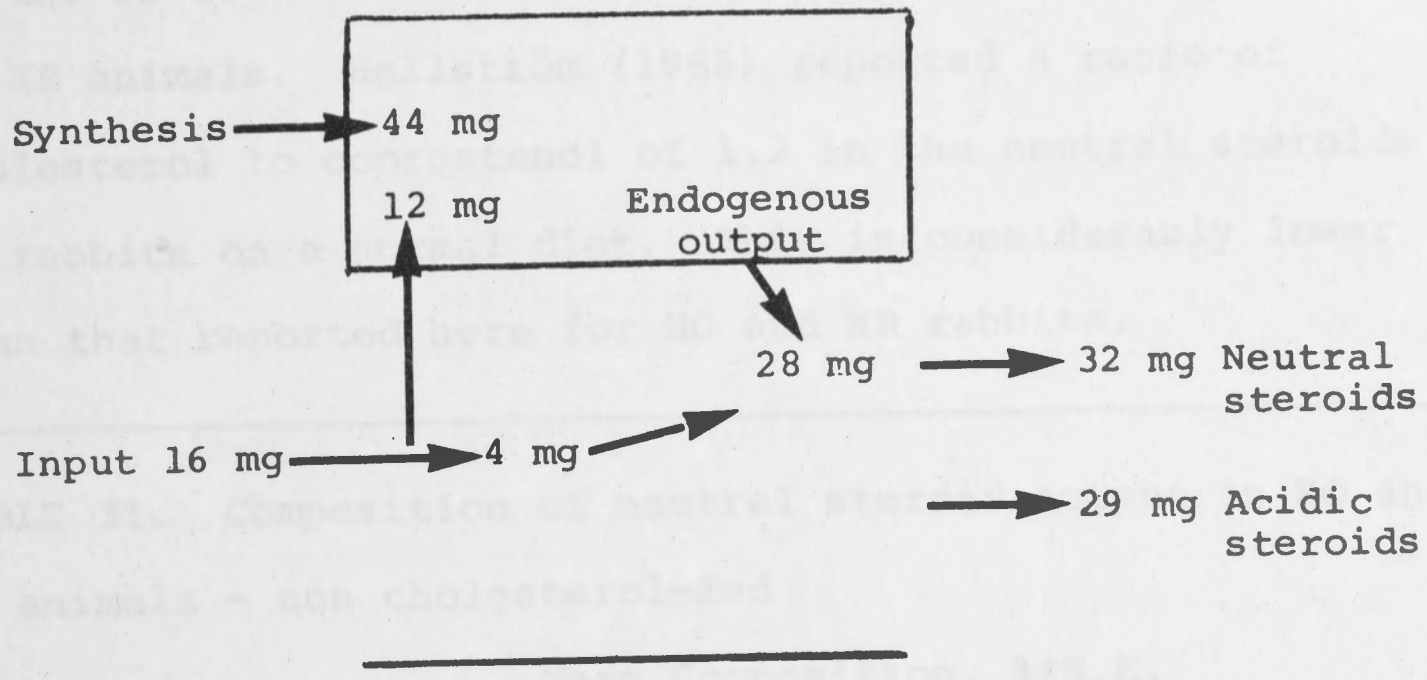
HO	Absorbed cholesterol, mg/day	Plasma cholesterol derived from absorbed cholesterol, %	Cholesterol synthesis, mg/day	Endogenous neutral steroid output, mg/day	Cholesterol entry mg/day
1	14	12.1	63	25	119
2	14	15.3	30	27	89
3	8	13.5	45	27	60
4	12	11.8	50	32	99
5	10	14.9	33	25	65
Mean±S.E.	11.6±1.16	13.5±0.71	44.2±5.98	27.0±1.30	86.4±10.92
HR					
1	14	15.6	37	14	87
2	13	13.5	46	19	96
3	15	19.8	46	27	76
4	14	14.9	35	30	95
5	15	13.5	34	26	114
6	14	11.0	53	31	125
Mean±S.E.	14.2±0.31	14.7±1.20	41.8±3.11	24.5±2.71	98.8±7.30

Absorption of dietary cholesterol was significantly higher ($P<0.05$) for HR animals being 88% of the dietary intake. This is reflected in the higher daily cholesterol entry seen in HR rabbits (99±7.3 mg/day mean±S.E.) when compared with HO rabbits (86±10.9 mg/day). Cook, Kliman and Fieser (1954) report that rabbits fed 1% cholesterol with 16.6% olive oil absorb 90% of the daily cholesterol corresponding to 250 mg/kg body weight per day. The animals reported here consume a diet containing 0.022% cholesterol and 3.5-4.5% fat and absorb approximately 4.3 mg/kg body weight per day (assuming a mean body weight of 3 kg and a mean absorption of 13 mg/day). The fraction of the plasma cholesterol derived from absorbed cholesterol was similar for both HO (13.5±0.71%, mean±S.E.) and HR animals (14.7±1.20%). Endogenous neutral steroid output is higher, but not significantly so, in HO rabbits.

Cholesterol synthesis, as measured by the difference between input and total faecal steroid output, is similar for both HO (44.2±5.98 mg/day, mean±S.E.) and HR animals (41.8±3.11 mg/day). Thus there is very little difference between hypo-responder and hyper-responder rabbits when they are consuming a diet containing no added cholesterol. The output of endogenous neutral steroid is slightly higher in HO animals and more cholesterol enters the miscible pools per day in HR animals. Steroid balances for HO and HR rabbits fed a diet containing no added cholesterol are summarised in Figure 13.

The proportion of cholesterol and β-sitosterol present in the faeces as their respective 5β-ol and 5β-one

HO



HR

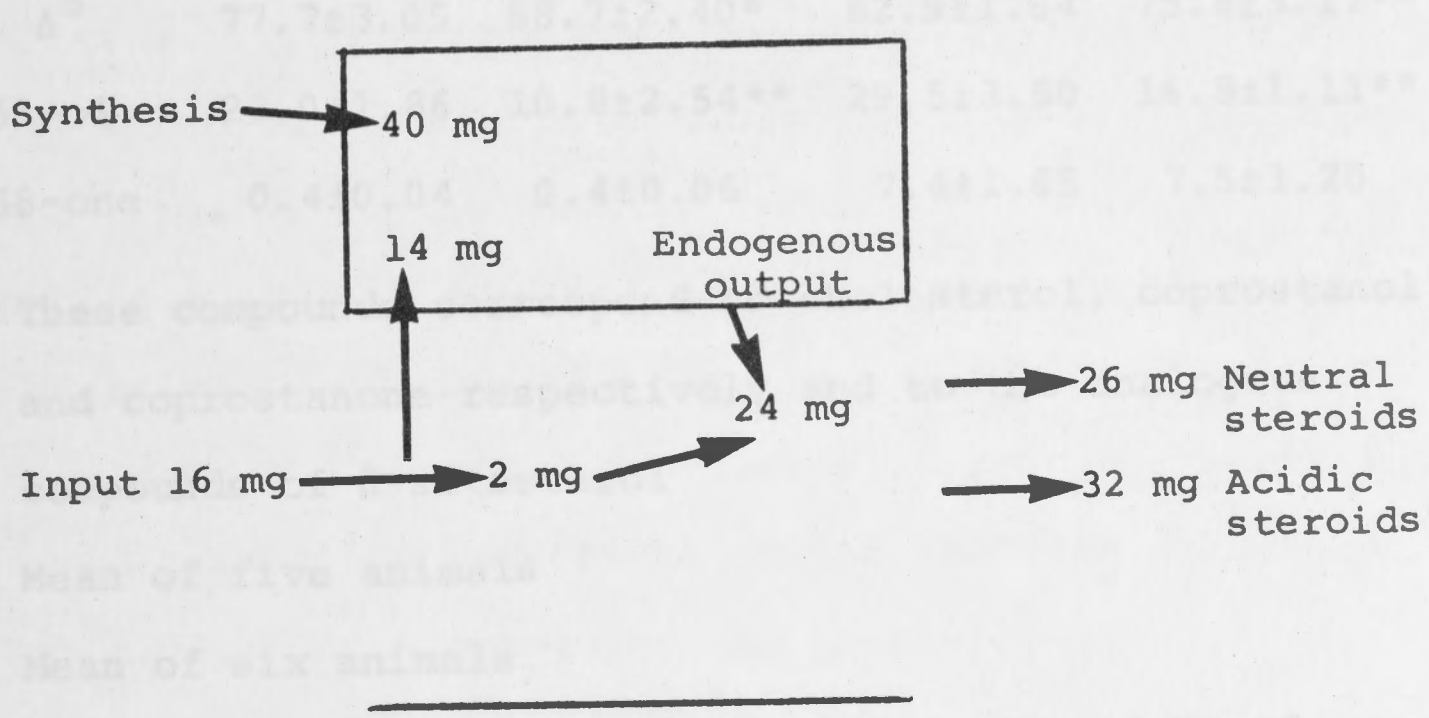


Figure 13. *Daily cholesterol balances in hypo-responder (HO) and hyper-responder (HR) rabbits consuming a diet containing no added cholesterol.*

metabolites are shown in Table 31. A reduction in output of the 5β-ol of cholesterol and β-sitosterol was observed in HR animals. Hellström (1965) reported a ratio of cholesterol to coprostanol of 1.2 in the neutral steroids of rabbits on a normal diet. This is considerably lower than that reported here for HO and HR rabbits.

TABLE 31. Composition of neutral steroid output in HO and HR animals - non cholesterol-fed

Neutral steroids	Mean Composition, %±S.E.			
	Cholesterol[a]		β-sitosterol[a]	
	HO[b]	HR[c]	HO	HR
Δ^5	77.7±3.05	88.7±2.40*	62.9±1.64	75.8±3.17**
5β-ol	23.0±1.86	10.8±2.54**	29.5±3.50	16.8±1.11**
5β-one	0.4±0.04	0.4±0.06	7.4±1.65	7.5±1.20

a These compounds correspond to cholesterol, coprostanol and coprostanone respectively and to the analogous compounds of β-sitosterol

b Mean of five animals

c Mean of six animals

Comparisons by Students' t-test between HO and HR of same group

* $P<0.05$ ** $P<0.01$

(iii) Steroid balance for HO and HR rabbits on a diet containing added cholesterol:

Data obtained from weeks 11-13 of the experiment (3rd to 5th week of cholesterol feeding) were pooled and the mean daily output of neutral and acidic steroids from

the five HO and six HR animals were calculated (Table 32). Comparison of the results shown in Table 28 and in Table 32 show that both neutral and acid steroid output is increased with cholesterol feeding. Again, the output of neutral steroids is higher,but not significantly,for HO animals when compared with HR animals. Hellström (1965) reports that rabbits fed a 1% (w/w) cholesterol diet excrete 520 mg neutral steroid in their faeces per day. The animals reported here are producing only half that quantity on a proportional basis (dietary cholesterol = 0.31%, w/w). However, output of acidic steroids is considerably higher on a proportional basis than that reported by Hellström (1965) for the output of deoxycholic acid. Total output of faecal steroids derived from cholesterol is significantly higher ($P<0.05$) for HO animals (Table 32). However, total output is less than the daily dietary input for both HO and HR animals and thus the pool size must still be increasing. The specific radioactivities of the neutral and acidic steroids and plasma cholesterol are presented in Table 33. Daily output of radioactivity for HO (141±4.3 mμCi, mean±S.E.) and HR (124±9.8 mμCi) animals is more than the input (100.1 mμCi).

The mean plasma cholesterol specific radioactivity approximates the dietary cholesterol specific radioactivity. Thus the percentage of plasma cholesterol derived from absorbed cholesterol (Table 34) is high in both HO (87.9±3.45%, mean±S.E.) and HR rabbits (101.6±1.81%). This result indicates that the rate of synthesis is negligible in HR animals and very low in HO animals.

TABLE 32. Balance of neutral and acidic steroids in HO and HR rabbits - cholesterol-fed

		Balance mg/day		
		Faecal steroids		
HO	Dietary[a] cholesterol	Neutral	Acidic[b]	Total
1	217	127	19	146
2	217	57	99	156
3	217	81	74	155
4	217	106	47	153
5	217	73	41	114
Mean±S.E.	-	88.8±12.4	56.0±13.87	144.8±7.89
HR				
1	217	53	68	121
2	217	48	34	82
3	217	52	42	94
4	217	65	67	132
5	217	69	28	97
6	217	96	55	151
Mean±S.E.	-	63.0±7.26	49.0±6.92	113.0±10.7*

a This is composed of 16 mg present in the diet plus 201 mg added to the diet

b See footnote b to Table 28

Comparisons by Students' t-test between HO and HR of same group

*<P 0.05

TABLE 33. Specific radioactivity of neutral and acidic steroids in HO and HR animals - cholesterol-fed

	Specific activity of steroids μCi/gC[a]			
	Dietary cholesterol	Plasma cholesterol	Faecal steroids	
HO			Neutral	Acidic
1	0.551	0.52	0.87	2.098
2	"	0.44	0.87	0.790
3	"	0.44	0.87	1.078
4	"	0.50	0.58	1.643
5	"	0.52	1.28	1.067
Mean±S.E.	-	0.48±0.018	0.89±0.112	1.34±0.235
HR				
1	0.551	0.54	1.00	0.688
2	"	0.59	1.51	0.835
3	"	0.53	1.44	1.382
4	"	0.57	1.00	1.321
5	"	0.57	0.98	1.536
6	"	0.54	0.97	1.020
Mean±S.E.	-	0.56±0.010	1.15±0.103	1.13±0.137

[a] See footnote a to Table 29

TABLE 34. Parameters of cholesterol metabolism in HO and HR rabbits - cholesterol-fed[a]

	Plasma cholesterol derived from absorbed cholesterol, %	Cholesterol synthesis, mg/day
HO		
1	95.1	-71
2	79.5	-61
3	80.1	-62
4	90.2	-64
5	94.9	-113
Mean±S.E.	87.9±3.45	-74±9.86
HR		
1	98.4	-96
2	108.0	-135
3	96.8	-123
4	104.4	-85
5	103.8	-120
6	98.2	-102
Mean±S.E.	101.6±1.81**	-110.2±7.7*

[a] Calculated using equations in Table 28

Comparisons by Students' t-test between HO and HR rabbits of same group

* $P<0.05$ ** $P<0.01$

Synthesis, as calculated by output minus input, is negative for both HO (-74±9.9 mg/day, mean±S.E.) and HR animals (-110±7.7 mg/day) indicating that the pool size is expanding and at a significantly greater rate ($P<0.01$) for HR animals (Table 34). Obviously, the relationship of output minus input equalling synthesis is invalid at this time and the relationship should be output minus input equals synthesis minus change in pool size. Clearly, these animals cannot be considered to be in a metabolic steady state with regard to either mass balance or isotope balance.

Because the plasma cholesterol specific radioactivity approximates the dietary cholesterol specific radioactivity, the divisor in equation 2 (Table 26) is very small and this produces obviously erroneous (large or negative) values for cholesterol absorption and hence entry and endogenous neutral steroid output. Therefore, it has not been possible to calculate these parameters of cholesterol metabolism in cholesterol-fed HO and HR rabbits.

The conversion of cholesterol to coprostanol is reduced after cholesterol feeding (Table 35). Hellström (1965) reports a ratio of cholesterol to coprostanol of 3.4 in rabbits after cholesterol feeding. The rabbits reported here have a ratio three to six times that value. It is of interest that the HR rabbits exhibit significantly less ($P<0.01$) conversion of cholesterol to coprostanol during the period on the diet containing 16 mg/day of cholesterol (Table 31).

TABLE 35. Composition of neutral steroid output in HO and HR animals - cholesterol-fed

Neutral steroids	Mean Composition, %±S.E.			
	Cholesterol[a]		β-sitosterol[a]	
	HO[b]	HR[c]	HO	HR
Δ^5	89.7±1.66	93.3±1.58	79.3±0.88	76.0±4.16
5β-ol	10.1±1.77	5.8±1.38	15.7±0.67	15.6±2.67
5β-one	0.2±0.04	0.3±0.06	5.7±1.20	8.5±1.50

a See footnote a to Table 31

b Mean of five animals

c Mean of six animals

(b) Discussion:

The principal differences between hypo-responder and hyper-responder rabbits are only seen during the period of cholesterol feeding when there is a considerable difference in plasma cholesterol concentration. The results obtained in non cholesterol-fed rabbits are in broad agreement with those reported by earlier workers (Cook and Thomson, 1951; Cook et al., 1954; Hellström, 1962, 1965). Studies on hypo- and hyper-responder cholesterol-fed squirrel monkeys showed that acidic steroid excretion increased more rapidly in HO animals than HR animals (Lofland, Clarkson, St Clair and Lehner, 1972) and neutral steroid excretion was unchanged. Cholesterol balance studies carried out in rats (Wilson, 1964) and dogs (Pertsemlidis, Kirchman and Ahrens, 1973) also showed increased acidic steroid output during cholesterol feeding. This is the

opposite to what is happening in HO and HR rabbits where neutral steroid output is higher in HO than HR rabbits and acidic steroid excretion is similar for both HO and HR animals, although higher than in the non cholesterol-fed state. A similar situation exists in man where increased absorption of dietary cholesterol produces increased output of endogenous faecal neutral steroids (Quintão, Grundy and Ahrens, 1971) but little change in acidic steroid output. A recent report concerning hypo- and hyper-responder pigeons suggests that HO pigeons excrete more cholesterol daily when on a diet containing 1% cholesterol and 10% lard than do HR pigeons (Wagner and Clarkson, 1974).

Absorption of ingested cholesterol was higher for HR rabbits than HO rabbits, amounting to 88% of ingested cholesterol during the period in which the animals consumed the diet containing 16 mg/day of cholesterol. During the period that the rabbits received 217 mg/day of cholesterol, the proportion of plasma cholesterol derived from the diet was significantly higher in the HR animals. This is consistent with increased absorption or lower synthesis in such animals. However, enzymes studies show no difference in the rate of cholesterol synthesis between HO and HR animals (see below). As no estimate of endogenous neutral steroid output can be made, it is not possible to determine if the significantly increased output of total faecal steroids in HO animals is the result of decreased absorption or increased endogenous excretion. In humans, absorption is about 50% when intake is small and 25-30% on larger intakes of cholesterol (Quintão et al., 1971).

Absorption of cholesterol in the rat amounts to 71% on a high cholesterol diet (30 mg/day, Wilson, 1964). Novitskii (1971) reports that absorption in rabbits "resistant" to dietary cholesterol does not differ from that found in normal rabbits.

The contribution of dietary cholesterol to plasma cholesterol during the period on 217 mg/day of cholesterol amounted to 100% in HR rabbits and 88% in HO rabbits. This is considerably more than that seen in HO and HR squirrel monkeys (60%, Lofland et al., 1972), man (less than 50%, Grundy and Ahrens, 1969), baboon and rhesus monkey (60%, 69%, Eggen, 1974) but similar to that seen in the rat (90%, Grundy and Ahrens, 1969).

Thus, in summary, hyper-responder rabbits when fed cholesterol develop an increased hypercholesterolaemia when compared with hypo-responder rabbits. This seems to be brought about by the ability of the HO animals to excrete more total faecal steroids derived from cholesterol. However, it is not possible to determine what proportion of the faecal neutral steroids represents unabsorbed dietary cholesterol.

3. Distribution of total cholesterol in HO and HR rabbits between plasma and liver and the relative contributions of cholesterol and cholesteryl ester.

From the cholesterol balance study, it was clear that HO and HR rabbits could not be distinguished biochemically prior to feeding the cholesterol containing diet. The possibility that the transport of cholesterol in the lipoproteins could differentiate between HO and HR

animals after cholesterol feeding has been discounted (see previously). The next step was to investigate the possibility that hypo-responder animals were better able to prevent cholesterol release from the liver. To investigate this possibility, ten HO and ten HR rabbits were fed the cholesterol containing diet (200 mg cholesterol per day) from age ten weeks for a period of three weeks. At the end of this period the animals were killed and plasma and liver cholesterol and cholesteryl ester estimated.

(a) Results and discussion:

The plasma total cholesterol for HR rabbits was significantly higher ($P<0.01$) than that for HO rabbits and this was reflected in the significantly higher ($P<0.05$) free cholesterol and cholesteryl ester seen in the HR rabbits (Table 36). As both free cholesterol and

---

TABLE 36. Plasma cholesterol concentrations in HO and HR rabbits - cholesterol-fed

	HO	HR
Total cholesterol, mg/dl	460±25.1	677±58.4**
Free cholesterol, mg/dl	133±9.3	203±23.5*
Cholesteryl ester, mg/dl	316±23.1	445±34.8**
Free/ester ratio	0.44±0.042	0.36±0.037

Results are mean±S.E., ten animals per group

Comparisons by Students' t-test between HO and HR of same group

* $P<0.05$ ** $P<0.01$

esterified cholesterol were raised in HR rabbits the ratio of free cholesterol to esterified cholesterol was similar for both HO (0.44±0.042, mean±S.E.) and HR rabbits (0.36± 0.037). Recovery of total cholesterol after TLC generally exceeded 95%. The ratio of free to esterified cholesterol is similar to that reported by Wang et al. (1953) for cholesterol-fed rabbits and is also unchanged from the non cholesterol-fed ratio for HO (0.46±0.023, n=3) and HR animals (0.35±0.061, n=5).

There were no differences in body weight between the two groups but liver weight was significantly higher ($P<0.001$) for HR animals (Table 37). The concentration of total cholesterol in the liver was significantly higher ($P<0.05$) in HO animals. However, because of the differences in liver weight, total cholesterol per liver was similar in HO and HR rabbits (Table 37). The total plasma-liver cholesterol pool, estimated by assuming a plasma volume of 42.3 ml/kg body weight for all rabbits (Aikawa, 1950), was significantly ($P<0.05$) increased in HR rabbits (Table 37).

The increased concentration of total cholesterol in the liver of HO animals is the result of increased concentration of cholesteryl ester and this is reflected in the altered ratio of free cholesterol and esterified cholesterol (Table 38). In the non cholesterol-fed state, free cholesterol accounts for about 80% of liver total cholesterol (Evans and Oleksyshyn, 1959) and it is the cholesteryl ester which appears to be the preferred form for liver storage after cholesterol feeding (Billiau, Evrard, Van den Bosch, Joossens and De Somer, 1963;

TABLE 37. Body weights, liver weights and liver cholesterol concentrations in HO and HR rabbits - cholesterol-fed

	HO	HR
Body weight, kg	2.12±0.032	2.03±0.046
Liver weight (wet), kg	70.0±1.9	94.0±5.0***
Liver total cholesterol concentration, mg/g	6.0±0.40	4.8±0.15*
Total liver cholesterol, mg	424±34.8	457±31.2
Plasma-liver cholesterol pool, mg	835±50.6	1035±74.2*

Results are mean S.E., ten animals per group. Results from liver are expressed on a wet weight basis

Comparisons by Students' t-test between HO and HR of same group

* P<0.05 *** P<0.001

TABLE 38. Free cholesterol and cholesteryl ester concentrations in the livers of HO and HR rabbits - cholesterol fed

	HO	HR
Free cholesterol, mg/g	2.0±0.11	2.1±0.08
Cholesteryl ester, mg/g	3.6±0.31	2.6±0.12**
Free/ester ratio	0.59±0.040	0.79±0.027***

Results are mean±S.E., ten animals per group. Results from liver are expressed on a wet weight basis
Comparisons by Students' t-test between HO and HR of same group
** P<0.01 *** P<0.001

Nestel, 1970). This is also true for HO and HR rabbits. Prior to cholesterol feeding, about 83% of liver cholesterol is unesterified (free/ester ratio=6.31±0.378 for HO animals, n=3 and 4.67±1.225 for HR animals, n=5) and after cholesterol feeding the amount of unesterified liver cholesterol falls to 44% in HR animals and 37% in HO animals (Table 38). Thus the hypo-responder rabbit stores more cholesterol as cholesteryl ester in the liver than does the hyper-responder rabbit. The report by Camejo et al. (1973) which states that increased concentrations of intracellular cholesterol leads to increased lipoprotein biosynthesis in rabbits may be important, if unesterified cholesterol is a precursor of lipoprotein. If this

speculation is correct, one would expect to see the increased plasma cholesterol concentration which is in fact observed in HR animals.

4. Enzyme assays and tissue ascorbic acid analyses in HO and HR rabbits.

Experiments were conducted to measure the rate of cholesterol synthesis in order to confirm the result obtained by the steroid balance study. As 7α-hydroxylase is the rate limiting enzyme in the conversion of cholesterol to bile acids, attempts were made to measure the activity of this enzyme. The rate of hepatic cholesteryl ester hydrolysis was measured because of the increased concentration of cholesteryl ester found in the livers of HO animals. In addition, the initial rate of plasma cholesterol esterification was measured because the enzyme lecithin-cholesterol acyltransferase (LCAT) is intimately involved in the maintenance of the plasma cholesterol concentration (Glomset, 1968; Schumaker and Adams, 1969).

Although it has been shown that the rabbit is capable of synthesising ascorbic acid, there is an increased requirement for this vitamin during cholesterol feeding (Ginter, 1970, 1972). Rabbits "resistant" to hypercholesterolaemia have also been shown to have increased concentrations of ascorbic acid in the adrenals (Novitskii, 1971). For these reasons, the concentrations of ascorbic acid in whole blood, liver and adrenals were also measured in HO and HR rabbits. In addition plasma and liver cholesterol concentrations were measured.

Two experiments were conducted. In experiment 1 cholesterol synthesis, cholesteryl ester hydrolase activity and the concentrations of ascorbic acid in blood, liver and adrenals were measured in six HO and six HR rabbits after three weeks on the cholesterol containing diet (200 mg/day of cholesterol). In addition to the above assays, LCAT activity was measured both before and after cholesterol feeding in experiment 2 on five HO and five HR animals. Preliminary experiments to measure 7α-hydroxylase activity were unsuccessful and so the activity of this enzyme was not measured in either experiments 1 or 2.

(a) Results:

(i) Body weight, liver weight and cholesterol concentrations in plasma and liver of HO and HR rabbits used for the enzyme experiments:

The mean body weights and liver weights for the HO and HR rabbits used in the two experiments are shown in Table 39. Body weight was similar for both HO and HR rabbits. As reported previously, liver weight was significantly higher ($P<0.01$) in the HO rabbits of experiment 1. However, in experiment 2, the liver weights of HO animals were not significantly different from those of the HR animals (Table 39). Pooling of the data from experiments 1 and 2 resulted in significantly higher ($P<0.01$) liver weights for HO animals (136±5.0 g, mean± S.E., n=11) when compared with HR animals (111±5.4 g, n=11). The mean plasma and liver total, free and esterified cholesterol concentrations for the rabbits in experiments 1 and 2 are also contained in Table 39.

As expected plasma total, free and esterified cholesterol concentrations are significantly higher in HR animals. As noted previously in the balance study, the magnitude of the cholesterolaemia produced after three weeks on the cholesterol diet (200 mg of cholesterol per day) is not as great as that seen during the breeding program. Again, these animals are older (between 20 and 30 weeks of age) than those tested during the breeding program at ten weeks of age. Liver total cholesterol concentration is significantly ($P<0.05$) increased in HO rabbits and this increase is in the cholesteryl ester content of the liver. Although the mean ratio of free cholesterol to cholesteryl ester is higher in HR animals the difference is not significant even when the data from experiments 1 and 2 are pooled (HO ratio=0.54±0.061, n=11; HR ratio=0.67± 0.050, n=11). With the exception of this last point, the results obtained with these two groups of animals confirm the findings of the preceding section.

(ii) Ascorbic acid in blood, liver and adrenals:

The mean ascorbic acid concentrations in blood, liver and adrenals for HO and HR rabbits after three weeks on the cholesterol-containing diet are summarised in Table 40. Adrenal ascorbic acid concentration was not estimated in experiment 2. It can be seen that, although HO rabbits have a slightly higher blood ascorbic acid concentration than HR rabbits, the difference is not statistically significant. The values obtained are similar to those reported by Harris, Constable, Howard and Leader (1956) for non cholesterol-fed rabbits.

TABLE 39. Body weights, liver weights and plasma and liver cholesterol concentrations of HO and HR rabbits used for the enzyme experiments

	Experiment 1		Experiment 2	
	HO	HR	HO	HR
No. of animals	6	6	5	5
Body weight, kg	2.79±0.126	2.81±0.116	2.55±0.255	2.37±0.064
Liver weight (wet), g	128±5.3	108±2.9**	144±8.0	114±11.9
Plasma cholesterol, mg/dl				
Total[a]	361±41.7	533±21.0**	340±20.2	472±25.6**
Free	108±13.9	151±7.7*	82±5.1	128±13.7*
Ester	258±30.2	379±21.3**	245±15.7	326±12.8**
Liver cholesterol, mg/g				
Total[a]	8.1±0.43	6.7±0.23*	5.9±0.64	4.0±0.37*
Free	2.5±0.27	2.7±0.14	2.2±0.42	1.6±0.09
Ester	5.6±0.46	4.2±0.17*	3.6±0.32	2.4±0.31*

Results are mean±S.E.

[a] The sum of the concentrations of free and esterified cholesterol does not equal the total cholesterol because the total cholesteral was estimated prior to TLC

Comparisons by Students' t-test between HO and HR of same group within each experiment

* $P<0.05$ ** $P<0.01$

TABLE 40. Ascorbic acid concentrations in the tissues of cholesterol-fed HO and HR rabbits

	Ascorbic acid concentration, mean±S.E.			
	Experiment 1		Experiment 2	
	HO	HR	HO	HR
No. of animals	6	6	5	5
Whole blood, mg/dl	1.29±0.186	0.98±0.135	0.93±0.062	0.87±0.092
Liver, mg/g	0.17±0.006	0.17±0.005	0.11±0.078	0.11±0.094
Adrenal, mg/g	0.96±0.140	0.82±0.090	-	-

The ascorbic acid concentrations in liver were not different for HO and HR rabbits and were similar to those reported by Ginter (1970) for cholesterol-fed rabbits. They were not appreciably different from those reported for non cholesterol-fed rabbits (Harris et al., 1956). In this laboratory values of 0.14±0.013 mg/g for the liver ascorbic acid concentration of twelve non cholesterol-fed rabbits have been reported (S. D. Turley, personal communication). When the difference in liver weights between HO and HR rabbits in experiment 1 is taken into account, the total ascorbic acid per liver is significantly higher ($P<0.05$) in HO animals (21.8±1.29 mg, mean±S.E., n=6) when compared with HR animals (17.9±0.54 mg, n=6). However, if the data from experiments 1 and 2 are pooled the total ascorbic acid per liver is not significantly higher in HO animals (18.7±1.39 mg, mean±S.E., n=11) when compared with HR animals (15.5±1.19 mg, n=11).

The ascorbic acid concentration in the adrenals of rabbits "resistant" to dietary cholesterol has been reported to be higher than that found in non-resistant animals (Novitskii, 1971). This is not so for the HO and HR rabbits reported here (Table 40). Adrenal weights were similar for HO (562±23.4 mg, mean±S.E., n=6) and HR animals (572±72.0 mg, n=6).

(iii) Hepatic cholesterol synthesis:

The results of estimates of cholesterol synthesis in cholesterol-fed rabbits as measured by the percent incorporation of 1-^{14}C acetate into digitonin-precipitible steroids by liver slices are summarised in Table 41.

TABLE 41. Incorporation of 1-^{14}C acetate into digitonin precipitable steroids by liver slices in cholesterol-fed HO and HR rabbits

	Experiment 1		Experiment 2	
	HO	HR	HO	HR
No. of animals	6	6	5	5
Acetate incorporation[a], %	0.03±0.007	0.03±0.003	0.06±0.016	0.07±0.032

Results are mean±S.E.

[a] 200 mg liver slices incubated for 2 hours at 37°C with 0.4 µCi acetate-1-^{14}C.

Assays for individual animals were performed in triplicate and results generally agreed within 5%. As can be seen from Table 41 the percent incorporation of 1-^{14}C-acetate is extremely low and the values are similar for both HO and HR animals. It was necessary to count the radioactivity of the digitonin precipitates for a considerable time (usually 50 minutes) to obtain a counting accuracy of 2%, as the counts per minute rarely exceeded four times the background rate. Incorporation of 1-^{14}C acetate for non cholesterol-fed animals was similar for both HO (0.27± 0.092%, mean±S.E., n=3) and HR rabbits (0.20±0.067%, n=3). These values are an order of magnitude less than those reported by Carroll (1971) for non cholesterol-fed rabbits. However, Carroll used more liver (750 mg) and a longer time of incubation (3 hours) and this may account for the differences. In this laboratory non cholesterol-fed

rabbits from the general colony have similar rates of synthesis (0.28 0.060%, n=12) to the HO and HR animals (S. D. Turley, personal communication). Gould and Taylor (1950) reported that dietary cholesterol suppressed hepatic synthesis to a few percent of the non cholesterol-fed rate. In cholesterol-fed HO and HR animals hepatic synthesis is reduced to about 10% of that seen in non cholesterol-fed animals. There is, however, no difference between HO and HR animals. Novitskii (1971) also reports no difference in hepatic cholesterol synthesis between those rabbits "resistant" and those susceptible to dietary cholesterol.

(iv) Hepatic cholesteryl ester hydrolase:

A preliminary experiment with five rabbits fed the cholesterol containing diet (200 mg of cholesterol per day) for three weeks established the linearity of the enzymic cholesteryl ester hydrolysis for the first sixty minutes of incibation (Figure 14). For both experiments 1 and 2 an incubation time of thirty minutes was used. In Table 42 are listed the mean protein and cholesteryl ester concentrations of the 105,000 g supernatant and the rate of cholesteryl ester hydrolysis expressed as percent conversion to cholesterol and as cholesteryl ester formed per mg protein per thirty minutes. Non-enzymic hydrolysis of cholesteryl ester was similar for HO and HR animals in experiment 1 (HO=0.88±0.104%, HR=0.86±0.072%, mean±S.E.) and experiment 2 (HO=0.24±0.024%, HR=0.26±0.018%). From Table 42 it can be seen that there are no differences between HO and HR animals in their ability to hydrolyse cholesteryl ester. Redgrave (1973) reports that cholesterol-fed rabbits

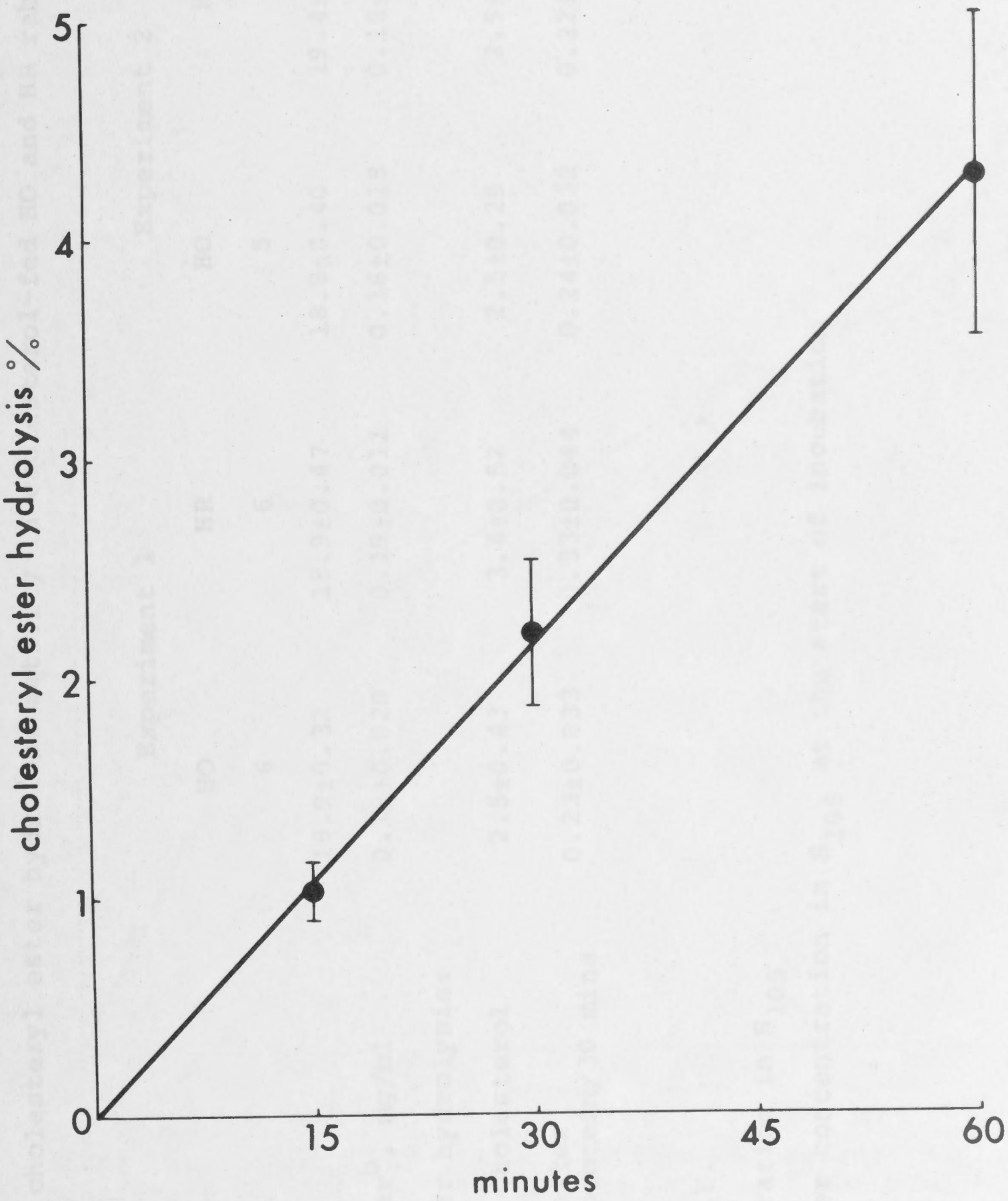


Figure 14. *Rate of cholesteryl ester hydrolysis in rabbit liver.* The points represent the mean % hydrolysis of five cholesterol-fed rabbits with the bars representing one S.E. above and below the mean.

TABLE 42. Hepatic cholesteryl ester hydrolase activity in cholesterol-fed HO and HR rabbits

	Experiment 1		Experiment 2	
	HO	HR	HO	HR
No. of animals	6	6	5	5
Protein[a], mg/ml	18.9±0.32	18.9±0.47	18.8±0.40	19.4±0.27
Cholesteryl ester[b], mg/ml	0.19±0.020	0.19±0.011	0.16±0.018	0.18±0.014
Cholesteryl ester hydrolysis:				
% conversion to cholesterol	2.5±0.43	3.4±0.52	2.5±0.29	2.5±0.26
µg cholesteryl ester hydrolysed/mg protein/30 mins	0.23±0.033	0.33±0.044	0.24±0.032	0.22±0.029

Results are mean±S.E.

a Protein concentration in S_{105}

b Cholesteryl ester concentration in S_{105} at the start of incubation

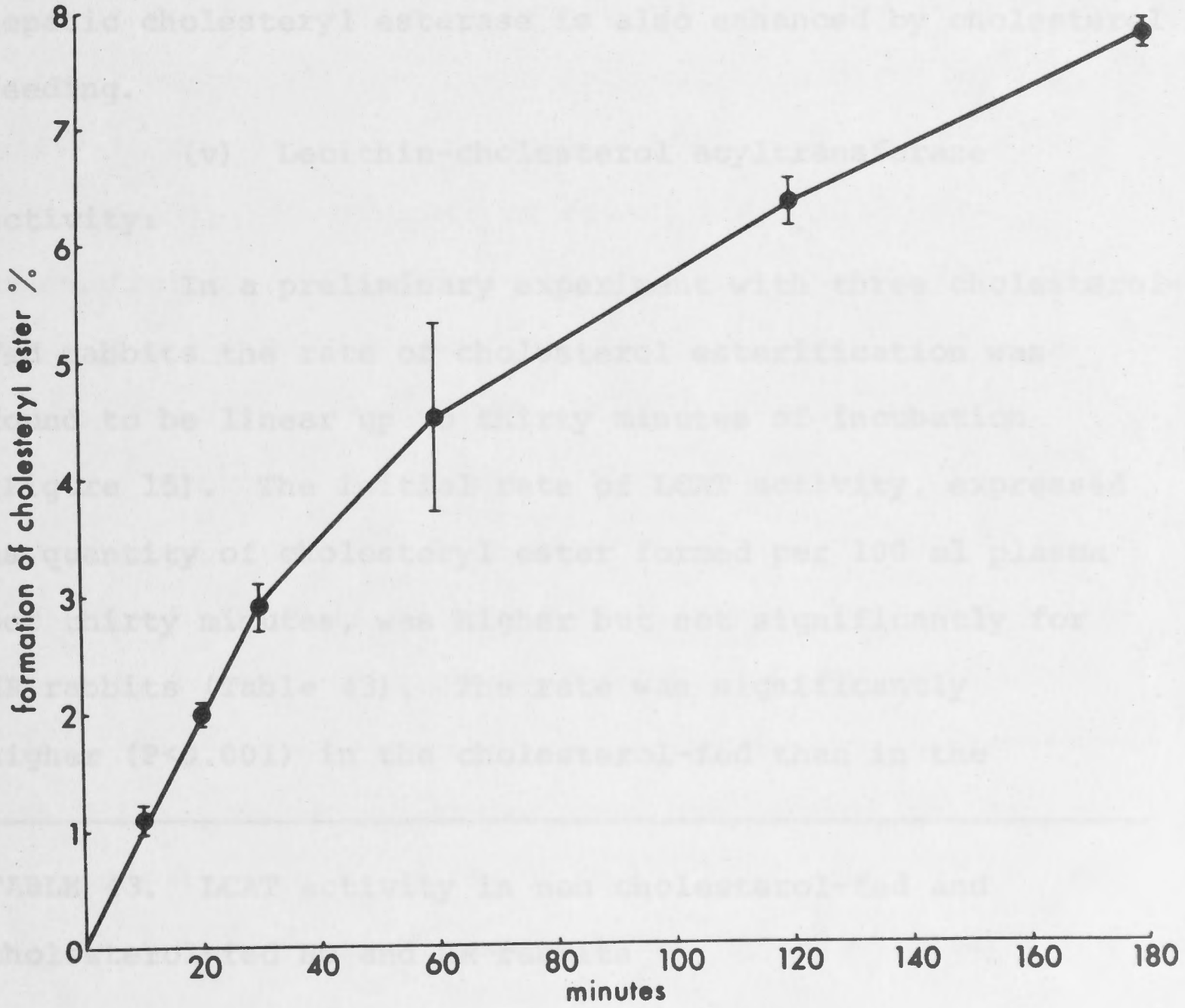


Figure 15. *Activity of plasma lecithin-cholesterol acyltransferase in rabbits.* The points represent the mean % formation of cholesteryl ester of three cholesterol-fed rabbits with the bars representing one S.E. above and below the mean.

have enhanced hepatic hydrolysis of cholesteryl ester when compared with non cholesterol-fed rabbits and cholesterol-fed rats. These observations were made indirectly from the clearance of isotopically labelled thoracic duct lymph cholesterol by the liver and not by direct enzyme assay. Felt and Benes (1970) have also shown that in rabbits, hepatic cholesteryl esterase is also enhanced by cholesterol feeding.

(v) Lecithin-cholesterol acyltransferase activity:

In a preliminary experiment with three cholesterol-fed rabbits the rate of cholesterol esterification was found to be linear up to thirty minutes of incubation (Figure 15). The initial rate of LCAT activity, expressed as quantity of cholesteryl ester formed per 100 ml plasma per thirty minutes, was higher but not significantly for HR rabbits (Table 43). The rate was significantly higher ($P<0.001$) in the cholesterol-fed than in the

---

TABLE 43. LCAT activity in non cholesterol-fed and cholesterol-fed HO and HR rabbits

	Non cholesterol-fed		Cholesterol-fed	
	HO	HR	HO	HR
No. of animals	5	5	5	5
Rate of esterification[a]	0.76±0.033	0.86±0.063	1.45±0.070	1.63±0.048

Results are mean±S.E.

[a] mg cholesteryl ester/30 mins/100 ml substrate

non cholesterol-fed animals. This may, however, be an artefact of the assay system as Raz, Kummerow and Nishida (1969) point out that increased concentrations of lipoprotein cholesterol result in increased rates of esterification by LCAT. Although the proportions of substrate plasma to "enzyme" plasma were 10:1, sufficient cholesterol was present in the "enzyme" plasma to increase the free cholesterol concentration in the incubation mixture for the assay in cholesterol-fed rabbits (Table 44). A similar relationship between rate of esterification and the concentration of free cholesterol in the plasma has also been shown for a number of species, including the rabbit, by Lacko, Rutenburg and Soloff (1974). In view of this, it is perhaps surprising that the cholesterol-fed HR animals do not show an increased rate of esterification when compared with the HO animals as they have a higher free cholesterol concentration. However, the majority of the substrate in

---

TABLE 44. Concentrations of free cholesterol in the incubation mixture for the assay of LCAT activity in non cholesterol-fed and cholesterol-fed HO and HR rabbits

	Non cholesterol-fed		Cholesterol-fed	
	HO	HR	HO	HR
No. of animals	5	5	5	5
Free cholesterol concentration, mg/dl	4.4±0.25	5.2±0.37	11.1±0.53	12.4±0.29

Results are mean±S.E.

the assay system is supplied by the pooled heat-inactivated rabbit plasma and this may have sufficiently diluted the additional free cholesterol supplied by the enzyme plasma to mask the difference in free cholesterol concentration between HO and HR animals (see Table 44). Hashimoto and Dayton (1971) report no increase in the rate of LCAT activity between non cholesterol-fed and cholesterol-fed rabbits when the initial substrate concentration is fixed.

Despite the problems associated with the free cholesterol concentration of the substrate between non cholesterol-fed and cholesterol-fed animals, within each feeding group there are no differences in plasma LCAT activity between HO and HR rabbits.

(vi) Cholesterol 7α-hydroxylase activity:

Preliminary experiments to determine the activity of cholesterol 7α-hydroxylase in rabbit liver failed to detect any activity above the background level of autoxidation taking place using either the method of Mitton et al. (1971) or Mitropoulos and Balasubramaniam (1972). Rat liver preparations prepared at the same time showed reasonable rates of conversion indicating the preparation of the S_{15} or S_{18} supernatant was adequate (Table 45). Cholestyramine feeding results in increased bile acid formation because of its ability to sequest the bile acids and interrupt the enterohepatic circulation. However, rabbits which had been consuming cholestyramine for four days prior to the enzyme assay did not show enhanced 7α-hydroxylase activity (Table 45). Incubations were normally carried out for thirty minutes at pH 7.4.

TABLE 45. Activity of cholesterol 7α-hydroxylase in rat and rabbit

Animal		7α-OH cholesterol produced, % Total	By autoxidation[a]
Rat	S_{15}[b]	3.7	0.23
	S_{18}[c]	1.7	0.23
Rabbit			
1. Non cholesterol-fed	S_{15}	0.68	0.57
	S_{18}	0.42	0.47
2. Cholesterol fed[d]	S_{18}	0.39	0.46
3. Cholestyramine fed[e]	S_{18}	0.24	0.17
	S_{15}	0.19	0.10
4. Cholestyramine fed, pH 6.5			
	S_{15}	0.24	0.17
5. Cholestyramine fed, pH 8.0			
	S_{15}	0.22	0.17
6. Cholestyramine fed	S_{15}		
incubate 40'		0.15	0.15
incubate 80'		0.27	0.28

[a] Boiled enzyme preparation

[b] S_{15} is the 15,000 g supernatant prepared by the method of Mitropoulos and Balasubramanian (1972)

[c] S_{18} is the 18,000 g supernatant prepared by the method of Mitton et al. (1971).

[c] Fed the cholesterol diet (200 mg cholesterol per day) for three weeks

[e] Fed cholestyramine (4% w/w) for four days prior to experiment

Prolonging the time of incubation increased the non-enzymic production of 7α-hydroxy-cholesterol and altering the pH did not produce an appreciable difference in the rate of hydroxylation of cholesterol (Table 45). Thus, this enzyme assay, as described in the literature for the rat, does not seem to work for rabbit hepatic 7α-hydroxylase.

(b) Discussion:

There is no difference between HO and HR rabbits in the concentrations of ascorbic acid in liver, blood and adrenals after cholesterol feeding. Ginter (1970) suggests that cholesterol feeding results in an accumulation of ascorbic acid in liver and adrenals of rats and rabbits and a depletion of tissue ascorbic acid in guinea pigs, animals which are unable to synthesise ascorbic acid. Novitskii (1971) reports an increased concentration of ascorbic acid in the adrenals, but not liver of rabbits "resistant" to dietary induced hypercholesterolaemia. These findings imply that an ability to increase ascorbic acid synthesis enables the animal to more readily metabolise the increased cholesterol intake. This does not seem to be a factor in differentiating the HO and HR rabbits. The effect of the addition of ascorbic acid to the diet of cholesterol-fed rabbits has been reported to have a hypocholesterolaemic effect (Ginter, Babala and Polonyora, 1970), a transitory hypocholesterolaemic effect (Zaitsev, Myasnikov, Kasatkina, Lobova and Sukasova, 1964), and no effect (Pool, Newmark, Dalton, Banziger and Howard, 1971). Clearly, the exact effect of ascorbic acid on cholesterol metabolism in rabbits is yet to be determined.

Hypo- and hyper-responder rabbits do not differ in the degree of inhibition of cholesterol synthesis induced by cholesterol feeding. The rate of synthesis is only 10% of that seen in the non cholesterol-fed state. Cholesteryl ester hydrolase activity was also similar in HO and HR rabbits despite the increased hepatic cholesteryl ester concentration in HO animals. The liver is the major source of plasma cholesteryl ester in cholesterol-fed rabbits (Rose, 1972) with LCAT only taking a minor role. LCAT activity in HR animals is slightly higher in both the non-cholesterol-fed state and the cholesterol-fed state. However, this increase is not significant. Further experiments designed in such a way as to enable the animals' own free cholesterol to be used as substrate (Lacko et al., 1974) may establish whether this trend is of importance.

7α-hydroxylase, the rate limiting enzyme in the formation of bile acids, could not be assayed in the rabbit because of methodological difficulties. The enzyme has been successfully assayed in the rat here and by other workers (see Table 1) and also in the guinea pig and the hamster (Kritchevsky, Tepper and Story, 1973; Schoenfield, Bonorris and Ganz, 1973). However, no reports of the assay of 7α-hydroxylase in rabbits are known at the present time.

In contrast to the results reported in section 3, HO animals had higher liver weights than HR animals. As a result of this, total cholesterol, free cholesterol and cholesteryl ester per liver is higher in HO than HR animals. This contradiction to the results reported earlier may be explained by the difference in age of these animals (20-30 weeks) compared to those reported in section 3 (10 weeks) or alternatively it may be an effect of their prior exposure to the cholesterol-containing diet at age ten weeks. However, the concentration of cholesteryl ester is always higher in HO animals when compared with HR animals.

CONCLUSION

In this thesis the observations of Redgrave and West (1972) on the differential effect of piperazine on cholesterol metabolism in male and female rabbits have been extended to castrated rabbits. Similar effects were produced in the castrated animals, i.e. in male rabbits hypercholesterolaemia was reduced but in females it was increased.

Hepatic microsomal cytochrome P-450 concentration was measured in these animals and was reduced in piperazine-treated males but not females. There appears to be no simple relationship between cytochrome P-450 and cholesterol metabolism.

During these experiments considerable individual variation in the cholesterolaemic response to dietary cholesterol was noted and this has been developed into a full study of the individual components of such variation in rabbits.

Rabbits, when consuming a diet containing no added cholesterol, exhibit quite wide variations in plasma cholesterol concentration. This variation can be assigned to two sources, a heritable component and an environmental component. In females, the heritability of the plasma cholesterol concentration is 62% and in males 22%. A recent report by Patton, Brown and Middleton (1974) has shown the heritability of plasma cholesterol concentration in pigeons to be 51%. These authors developed two strains, a line with reduced plasma cholesterol concentration and a line with increased plasma cholesterol concentration.

Most reports suggest a polygenic mode of inheritance of plasma cholesterol concentration in the animals studied. It is not possible to assess the mode of inheritance of the plasma cholesterol concentration in non cholesterol-fed rabbits because the design of the experiment was inappropriate for such an assessment.

Females have also been shown to have higher plasma cholesterol concentrations than males and this is significantly reduced during pregnancy. The plasma cholesterol concentration decreases with age in males but not females. In contrast to this, female dairy cattle exhibit an increase in plasma cholesterol concentration up to three years of age followed by a decrease over the ensuing ten years (Tumbleson and Hutcheson, 1971). Female rabbits also exhibit a slight seasonal variation which is not present in males. Female rats show a cyclical variation in plasma cholesterol concentration associated with the oestrus cycle (Fillios, Kaplan, Martin and Stare, 1958). This cannot explain the seasonal variation in female rabbits as they only ovulate after copulation. Seasonal variations have also been observed in four breeds of cattle, the plasma cholesterol concentration increasing during the winter months (O'Kelly, 1972, 1973).

The cholesterol-fed rabbit also exhibits considerable variation in the cholesterolaemia produced by cholesterol feeding and there is a correlation between the plasma cholesterol concentration before cholesterol feeding and the increase observed after cholesterol feeding. By selected breeding, it has been possible to

produce rabbits which differ markedly in their response to dietary cholesterol, the hypo-responder (HO) rabbit and the hyper-responder (HR) rabbit. The heritability of such traits is 50%. This value is in reasonable agreement with those reported for other species (Patton et al., 1974; Estep et al., 1969; Weibust, 1973; Eapen et al., 1971; Stufflebean and Lasley, 1969). There is no difference between sexes in the response of HO and HR rabbits to cholesterol feeding. However, it is possible that there may be an age effect as the cholesterolaemia is not so pronounced in older rabbits of both sexes. However, this factor was not examined in a systematic way and further experiments are necessary to clarify this point.

The response of the HO rabbit is dependent on a factor in their pre-weaning environment. HO animals raised on HR dams tend to resemble HR offspring raised on their natural dams. The response of HR rabbits is not altered by being raised on HO foster dams. Feigenbaum and Gaman (1967) report that the incidence of spontaneous aortic lesions in the offspring of Dutch rabbit dams is increased when they are raised on New Zealand White dams. Normally, rabbits of the Dutch strain have a relatively low incidence and rabbits of the New Zealand White strain have a high incidence of lesions. They conclude that there is a factor present in the milk of Dutch dams and absent in the milk of New Zealand White dams which affects the incidence of spontaneous aortic lesions. It has been observed that the milk of HR dams has a higher concentration of cholesterol and phospholipid than the milk of

HO dams. Thus, the character for hyper-response is independent of mothers' milk whilst the character for hypo-response is dependent on the cholesterol and possibly phospholipid concentration in the milk.

As some of the causes of variations in plasma cholesterol concentration in non cholesterol-fed and cholesterol-fed rabbits had been established, investigations were conducted to determine the differences between HO and HR rabbits. It was observed that the additional cholesterol contained in the plasma of HR rabbits is carried by very low density lipoprotein (VLDL) of $d<1.006$ and low density lipoprotein of $1.006<d<1.019$ (LDL_1). Cholesterol feeding in rabbits results in an increase in lipoproteins of $d<1.063$ (LDL and VLDL, Camejo et al., 1973; Camejo, Bosch and López, 1974). Chickens fed cholesterol also have increased concentration of VLDL (Kruski and Narayan, 1972). A similar situation exists for HO and HR rabbits. However, HO and HR rabbits do not differ in the percentage composition of the plasma lipoproteins as the increased cholesterol concentration in VLDL and LDL_1 in HR rabbits is accompanied by increased concentrations of phospholipid and protein.

The concentration of total cholesterol is higher in the livers of cholesterol-fed HO animals and this increase is in the concentration of cholesteryl ester. However, the weight of livers in HR animals is higher than that in HO animals and thus total cholesterol per liver is similar. There is no difference in the rate of hydrolysis of cholesteryl ester in the livers of HO or HR animals.

Hepatic cholesterol synthesis is suppressed to 10% of the non cholesterol-fed rate in both HO and HR rabbits. This is in contrast to the report of Adams et al. (1972) which concluded that hepatic synthesis was unaffected in New Zealand White and Dutch rabbits when fed a diet containing from 0.1-1% cholesterol. Dutch rabbits correspond to HO animals in their response to dietary cholesterol and New Zealand White rabbits correspond to HR animals. These authors did not detect any differences in the ability of these animals to absorb, catabolise or excrete cholesterol. However, HO rabbits excrete more total steroids derived from cholesterol than HR rabbits. This may be because absorption is less in HO rabbits or alternatively it may be because there is increased endogenous output of neutral steroids and possibly acidic steroids. Unfortunately, the availability of these specially bred HO and HR animals precluded the repeating of the balance studies to determine which of these alternatives was correct. Although hepatic cholesterol synthesis is suppressed equally in HO and HR rabbits after cholesterol feeding it is possible that intestinal cholesterol synthesis may be proceeding at different rates in these animals. This would seem to be unlikely considering that almost 100% of the plasma cholesterol is derived from absorbed cholesterol in HR rabbits and 90% in HO animals.

The significance of the reduced microbial degradation of cholesterol to coprostanol seen in HR animals is difficult to explain. However, this is unlikely to affect absorption as most microbial action takes place

in the large intestine and cholesterol is primarily absorbed in the upper third of the small intestine (McIntyre, Kirsch, Orr and Isselbacker, 1971; McIntyre and Isselbacker, 1973). A report by Pollack (1953) showed that feeding rabbits sitosterol (a mixture of α-, β- and γ-sitosterols) and cholesterol in the ratio of 6:1 prevented hypercholesterolaemia by preventing absorption. Dietary fibre has been shown to reduce bacterial dehydroxylation of bile salts in humans (Pomare and Heaton, 1973). However, as the rabbits were consuming similar diets this seems an unlikely explanation for the differences seen in HO and HR rabbits.

The key to the difference between HO and HR animals seems to be the plasma/liver partition of the cholesterol and with the increased faecal steroid output in HO animals. The ability of plasma lecithin-cholesterol acyl-transferase (LCAT) to esterify cholesterol is similar in both HO and HR animals. Thus this enzyme does not seem to be involved in the increased cholesterolaemia seen in HR animals. Although bile acid output is raised in cholesterol-fed animals, the major increase is in neutral steroid output which is even greater in HO than in HR animals. However, this increase failed to reach significance for the number of animals used. It is possible that with increased numbers of animals this trend could have been confirmed.

There is some evidence that cholesteryl ester is the substrate for the enzyme cholesterol 7α-hydroxylase (Boyd, 1962) and with the increased concentration of

cholesteryl ester in the livers of HO animals it is possible that increased bile acid production would aid in reducing the plasma cholesterol concentration. However it was not possible to measure the rate of hydroxylation of cholesterol by this enzyme. The balance of evidence, however, does not favour the idea that cholesteryl ester is the substrate. It is generally believed that the substrate is free cholesterol (Finagin, 1972; Mathe, D'Hollander and Chevallier, 1972). If this is correct, one would perhaps expect an increased rate of cholesteryl ester hydrolysis in HO rabbits and hence increased availability of free cholesterol for catabolism. Unfortunately the rates of ester hydrolysis are similar for both HO and HR animals.

Ascorbic acid is believed to be essential for the catabolism of cholesterol to bile acids in guinea pigs (Ginter, Cerven, Nemec and Mikus, 1971; Ginter, Nemec, Cerven and Mikus, 1973; Ginter, 1973). In cholesterol-fed HO and HR rabbits the concentrations of ascorbic acid in liver are similar although the ascorbic acid content of liver is higher in HO animals. On balance, it seems unlikely that enhanced acidic steroid output is the cause of reduced plasma cholesterol concentration in HO animals.

If absorption is similar in cholesterol-fed HO and HR rabbits, it is possible that the HO animal is better able to re-excrete absorbed cholesterol and hence not become as hypercholesterolaemic. Such a situation exists in man where increased absorption of dietary cholesterol produces increased output of endogenous faecal

neutral steroids (Quintao, Grundy and Ahrens, 1971). However, the difference may simply be one of absorption. In the non cholesterol-fed state HO animals absorb slightly less than HR animals and endogenous neutral steroid output is slightly higher.

In conclusion, the work in this thesis has established some of the causes of individual variation in plasma cholesterol concentration in both the non cholesterol-fed and cholesterol-fed rabbit. In addition, two strains of rabbits with reproducible responses to dietary cholesterol have been established. These animals will be extremely useful in studies of cholesterol metabolism or hyper- and hypo-cholesterolaemic agents as they will enable the researcher to use fewer animals per experiment because of the reduction in individual variation. A preliminary study of the possible biochemical differences between these two groups of animals has been undertaken. Essentially, the plasma/liver partition is different between cholesterol-fed HO and HR animals and the output of total faecal steroids derived from cholesterol is greater in HO animals. Further experiments are necessary to define the differences in more detail. However, during the period in which work for this thesis was undertaken sufficient specially bred animals were not available to carry out these further studies.

APPENDIX 1

DETERMINATION OF QUENCH CORRECTION CURVES FOR LIQUID SCINTILLATION COUNTING

Bulk solutions of scintillator (0.5% w/v diphenyloxazole in toluene) containing known amounts of ^{3}H- or ^{14}C-hexadecane were prepared and aliquots (10.0 ml) of these solutions were accurately pipetted into scintillation vials. In order to obtain chemical quenching, increasing volumes (10-200 µl) of chloroform were added to the vials. For silica gel quenching, increasing areas of gel from a TLC plate were scraped into the vials. The samples were then assayed for radioactivity in three channels with windows set such that there was a wide ^{14}C channel, a narrow ^{14}C channel and a ^{3}H channel. The windows of the wide ^{14}C channel were set to maximise the efficiency of ^{14}C counting (counting rate as a percentage of the known disintegration rate) and to minimise the effects of quenching on the efficiency of counting. The windows of the narrow ^{14}C channel were set such that the spillover of ^{3}H counts was less than 1%. The windows of the ^{3}H channel were set, not only to maximise the efficiency of ^{3}H counting but also to reduce the spillover of ^{14}C counts into this channel during dual isotope (^{14}C and ^{3}H) counting. The windows of the external standard channels were set to give an optimum range of external standard ratios (ESR) for use with the range of quenching expected.

The spillover of ^{14}C counts into the ^{3}H channel was calculated as the ratio of ^{14}C counts in the narrow

^{14}C channel to the ^{14}C counts in the ^{3}H channel. The ESR and the efficiency of counting were noted for the range of quenched samples. The quench correction curves for the ^{3}H channel and the narrow ^{14}C channel were fitted to a curve with the following equation:

$$Y = A + B1(X) + B2(X^2) + B3(X^3)$$

where Y = efficiency of counting, %

and X = external standard ratio.

For dual label counting the spillover of ^{14}C counts into the ^{3}H channel at various levels of quenching was fitted to a similar curve with the following equation:

$$\log Y = A + B1(X) + B2(X^2) + B3(X^3)$$

where Y = spillover of ^{14}C counts into ^{3}H channel, %

and X = external standard ratio.

To determine the coefficients, B1, B2 and B3, matrix coefficients of the 4 x 3 matrix were calculated from the data using program MC1D (Table 46) and the equation solved using program SM1D (Table 47). Program SM1D was written by Mr H. R. Kinns (Programmer to the John Curtin School of Medical Research). The goodness of fit of the curve was checked by comparing the fitted points with the experimental points. A typical example for the calculation of the coefficients for a ^{3}H quench curve is shown in Table 48. Data files of the coefficients and intercepts for the three correction curves were prepared for the various types of quenching, i.e. silica gel and chemical quenching in the normal scintillator and chemical quenching in the normal scintillator to which methanol (1.0 ml) had been added (for counting of steroid digitonides). Such

data files are shown in Table 49. To determine the radioactivity present in a sample (as mμCi) the appropriate data tape was added to the general program L21D (Table 50) and the ESR and counts per minute for the sample entered from the keyboard console.

```
*L C MC1D
*W
C-FOCAL, U05

01.05 I (-SW)10.1
01.06 T !!"CALC OF MATRIX COEFFS FOR LS100 QUENCH CURVES",!
01.10 S SW=1;A "N",N,!;F I=1,N;A !,X(I),Y(I)
01.20 F I=1,N;D 5
01.30 F I=1,N;D 6
01.40 F I=1,N;D 7
01.50 F J=1,9;T !,%,A(J)
01.60 L D MC1D;L S MC1D
01.70 L C SM1D

05.10 S X2(I)=X(I)↑2;S X3(I)=X2(I)*X(I);S X=X+X(I);S Y=Y+Y(I)
05.20 S X2=X2+X2(I);S X3=X3+X3(I)

06.10 S X(I)=X(I)-X/N;S X2(I)=X2(I)-X2/N;S Y(I)=Y(I)-Y/N
06.20 S X3(I)=X3(I)-X3/N

07.10 S A(1)=A(1)+X(I)↑2;S A(2)=A(2)+X(I)*X2(I);S A(5)=A(5)+X2(I)↑2
07.20 S A(3)=A(3)+X(I)*X3(I);S A(4)=A(4)+X(I)*Y(I)
07.30 S A(6)=A(6)+X2(I)*X3(I);S A(7)=A(7)+X2(I)*Y(I)
07.40 S A(9)=A(9)+X3(I)*Y(I);S A(8)=A(8)+X3(I)↑2

10.10 A !,"X",A,"X2",B,"X3",C
10.20 S D=Y/N-A*X/N-B*X2/N-C*X3/N;T !,"INTERCEPT",%,!,D
10.30 T !,"CALCULATED   EXPTL"
10.40 F I=1,N;T !,%,Y/N+A*X(I)+B*X2(I)+C*X3(I),Y/N+Y(I)
10.50 E
10.60 D 1.6
10.70 T !!"END"!!!:Q
*
```

Table 46. Focal program MCID used to calculate the matrix coefficients for solving the equations of the quench curves for liquid scintillation counting.

```
*L C SM1D
*W
C-FOCAL, U01

01.02 T !"ROUTINE TO SOLVE MATRIX EQ. AX=B FOR X"!
01.04 T !"ENTER DIMENSION OF A, THEN"
01.05 T !"ENTER COEFF'S A(J,K)...A(J,N) AND B(J)"!
01.06 A L,!: S N=L-1: S I=-1
01.11 F K=0,N; S R(K)=K+1
01.12 F J = 0,N; T !: F K=0,L; A A(J+K*L)
01.14 S M=1E-6
01.16 F J=0,N; F K=0,N; D 4
01.17 S R(P)=0.
01.18 F K=0,L; S A(P+L*K)=A(P+L*K)/M
01.20 F J=0,N; D 5
01.22 S I=I+1
01.23 I (I-N) 1.14,1.26,1.14
01.26 F J=0,N; F K=0,N; D 7
01.28 F K=0,N; T !%2,"X("K,") ",%8.05, X(K)
01.29 L C MC1D

04.05 I (R(J)) 0, 4.3, 4.1
04.10 I (FABS(A(J+L*K)) - FABS(M)) 4.3;
04.20 S M=A(J+K*L)
04.22 S P=J; S Q=K
04.30 R

05.10 I (J-P) 5.2, 5.4, 5.2
05.20 S D=A(J+L*Q)
05.30 F K=0,L; S A(J+L*K)=A(J+L*K)-A(P+L*K)*D
05.40 R

07.10 I (FABS(A(J+K*L))-1E-6) 7.3,7.3,7.2
07.20 S X(K)=A(J+L*L)
07.30 R
*
```

Table 47. Focal program SMID used to calculate the coefficients of the quench curves used in liquid scintillation counting.

```
L C MC1D
*E
*G


CALC OF MATRIX COEFFS FOR LS100 QUENCH CURVES
N:9

:15.32 :43.35
:15.13 :42.79
:11.87 :40.12
:9.91 :37.33
:8.81 :34.87
:8.05 :32.98
:7.38 :31.25
:6.37 :29.41
:5.75 :26.44
 0.101226E+03
 0.218084E+04
 0.371487E+05
 0.168368E+03
 0.476690E+05
 0.821729E+06
 0.352292E+04
 0.143045E+08
 0.595351E+05
ROUTINE TO SOLVE MATRIX EQ. AX=B FOR X

ENTER DIMENSION OF A, THEN
ENTER COEFF'S A(J,K)...A(J,N) AND B(J)
:3

:101.226 :2180.84 :37148.7 :168.368
:2180.84 :47669 :821729 :3522.92
:37148.7 :821729 :14304500 :59535.1
X(  0)     5.96268
X(  1) -   0.25582
X(  2)     0.00330
X:5.96268 X2:-.25582 X3:.0033
INTERCEPT
-0.587158E-01
CALCULATED    EXPTL
 0.431135E+02 0.433500E+02
 0.430247E+02 0.427900E+02
 0.401931E+02 0.421200E+02
 0.371195E+02 0.373300E+02
 0.348733E+02 0.348700E+02
 0.330845E+02 0.329800E+02
 0.313392E+02 0.312500E+02
 0.283961E+02 0.284100E+02
 0.263960E+02 0.264400E+02

END
```

matrix coefficients

equation coefficients

$$Y = -0.59 + 5.96X - 0.26X^2 + 0.003X^3$$

Table 48. Calculation of coefficients of ^3H quench curve using programs MCID and SMID

```
*L C LM1D
*W
C-FOCAL, U01

01.05 T "LS100 Q CURVES FOR 1.0 MEOH/10 PPO/T0L .5%. 1/11/73"
01.10 S A(1)=11.1597;S A(2)=-17.5387;S A(3)=9.303
01.20 S B1(1)=2.66152:S B2(1)=.26334:S B3(1)=-.02501
01.30 S B1(2)=16.0245;S B2(2)=-1.97601:S B3(2)=.12594
01.40 S B1(3)=-2.1465;S B2(3)=.36388;S B3(3)=-.02388
*
*
*L C LS1D
*W
C-FOCAL, U01

01.05 T "LS100 Q CURVES FOR SILICA GEL PPO/T0L .5%. 1/2/74"
01.06 T !"3H ONLY"
01.10 S A(1)=.0755386;S A(2)=-17.5387;S A(3)=9.303
01.20 S B1(1)=8.22323;S B2(1)=-.59516;S B3(1)=.01807
01.30 S B1(2)=16.0245;S B2(2)=-1.97601;S B3(2)=.12594
01.40 S B1(3)=-2.1465;S B2(3)=.36388;S B3(3)=-.02388
*
```

Table 49. Data files of coefficients for quench curves produced by different agents or scintillation mixtures.

```
*L C L21D
*W
C-FOCAL, U01

01.05 T "LS100 Q CURVES FOR 10 PPO/T0L .5%. 1/11/73"
01.10 S A(1)=5.217;S A(2)=-10.5165;S A(3)=7.19673
01.20 S B1(1)=5.99655;S B2(1)=-.29236;S B3(1)=.00542
01.30 S B1(2)=10.1835;S B2(2)=-.43722;S B3(2)=.00525
01.40 S B1(3)=-.7373;S B2(3)=.05156;S B3(3)=-.0014
01.60 A !,"B'GD,NARROW14C,3H,WIDE14C",!,D1,D2,D3,
01.70 A !"CALC DUAL LABEL(1),3H(2),OR 14C(3) ?",I;I (-I)1.8;T !"END":Q
01.80 I (2-I)4.1,4.2;T !"3H+14C"
01.90 A !"N0",N;T !"N14C  CTS N3H   E.S.R.        3H MUC/SMPLE 14C"

02.10 I (1-I)6.1;A !,C,"  "C2,"  ",X
02.15 F J=1,3;S Y(J)=A(J)+(B1(J)+X*(B2(J)+B3(J)*X))*X
02.20 I (1-I)7.1;S R=(C-D1)*.045045/Y(2)
02.30 S R2=((C2-D2)-(C-D1)*FEXP(Y(3))/100)*.045045/Y(1)
02.50 T !,%8.06,"                                        ",R2,"    ",R
02.60 S M=M+1;I (M-N)2.1
02.70 S M=0;G 1.7

04.10 T !"WIDE 14C";G 1.9
04.20 T !"3H";G 1.9

06.10 A !,C;I (2-I)6.2;A "          ",X;G 2.15
06.20 S R=(C-D3)*.4953E-3;S R2=0;G 2.5

07.10 S R2=(C-D2)*.045045/Y(1);S R=0;G 2.5
*
```

Table 50. General Focal program, L2ID for determining radioactivity (as mμCi) under conditions of variable quenching and with dual label counting.

REFERENCES

ADAMS, W. C., E. M. GAMAN and A. S. FEIGENBAUM. Breed differences in the responses of rabbits to atherogenic diets. Atherosclerosis 16, 405-411 (1972).

ADEL, H. N., Q. B. DEMING and L. BRUN. Genetic hypercholesterolemia in rats. Circulation 40, III-I (1969).

AIKAWA, J. K. Fluid volumes and electrolyte concentrations in normal rabbits. Am. J. Physiol. 162, 695-702 (1950).

ANITSCHKOW, N. Über die Atherosclerose der Aorta beim Kaninchen and über derin Entstehungsbedingungen. Beitr. Path. Anat. 59, 306-348 (1914).

ARINGER, L. and P. ENEROTH. Studies on the formation of C_7-oxygenated cholesterol and β-sitosterol metabolites in cell-free preparations of rat liver. J. Lipid Res. 14, 563-572 (1973).

ATKIN, S. D., E. D. PALMER, P. D. ENGLISH, B. MORGAN, M. A. CAWTHORNE and J. GREEN. The role of cytochrome P-450 in cholesterol biogenesis and catabolism. Biochem. J. 128, 237-242 (1972).

BACK, P., B. HAMPRECHT and F. LYNEN. Regulation of cholesterol biosynthesis in rat liver: diurnal changes of activity and influence of bile acids. Archs. Biochem. Biophys. 133, 11-21 (1969).

BERGMAN, A. J. and C. W. TURNER. The composition of rabbit milk stimulated by the lactogenic hormone. J. biol. Chem. 120, 21-27 (1937).

BERGSTRÖM, S. and U. GLOOR. Studies on the 7α-hydroxylation of taurodesoxycholic acid in rat liver homogenates. Bile acids and steroids 18. Acta chem. Scand. 9, 34-38 (1955).

BILLIAU, A., E. EVRARD, J. VAN DEN BOSCH, J. V. JOOSSENS and P. DE SOMER. The fatty acid distribution in plasma,

liver and aorta of cholesterol-fed and triton-treated rabbits. Atherosclerosis 3, 222-232 (1963).

BORGSTROM, B. Studies on intestinal cholesterol absorption in the human. J. clin. Invest. 39, 809-815 (1960).

BORTZ, W. M. Noradrenalin-induced increase in hepatic cholesterol synthesis and its blockade by puromycin. Biochim. biophys. Acta 152, 619-626 (1968).

BOYD, G. S. Effect of Linoleate and estrogen on cholesterol metabolism. Fedn. Proc. Fedn. Amer. Soc. exp. Biol. 21. Suppl. 11, 86-92 (1962).

BRAGDON, J. H. Spontaneous atherosclerosis in the rabbit. Circulation 5, 641-646 (1952).

BROWN, H. B. Diets that lower blood cholesterol in man. In Proceedings of the second international symposium on atherosclerosis. Editor R. J. Jones, Springer Verlag, New York, Inc. 426-435 (1969).

BRUELL, J. H. Additive inheritance of serum cholesterol level in mice. Science 142, 1664-1666 (1963).

CAMEJO, G., V. BOSCH and A. LOPEZ. The very low density lipoproteins of cholesterol-fed rabbits. Atherosclerosis 19, 139-152 (1974).

CAMEJO, G., V. BOSCH., C. ARREAZA and H. C. MENDEZ. Early changes in plasma lipoprotein structure and biosynthesis in cholesterol-fed rabbits. J. Lipid Res. 14, 61-68 (1973).

CARROLL, K. K. Acetate incorporation into cholesterol and fatty acids by livers of fetal, suckling and weaned rats. Can. J. Biochem. 42, 79-86 (1964).

CARROLL, K. K. Plasma cholesterol levels and liver cholesterol biosynthesis in rabbits fed commercial or

semisynthetic diets with and without added fats or oils. Atherosclerosis 13, 67-76 (1971).

CARROLL, K. K., R. M. G. HAMILTON and G. K. MACLEOD. Plasma cholesterol levels in suckling and weaned calves, lambs, pigs and colts. Lipids 8, 635-640 (1973).

CLARKSON, T. B. Atherosclerosis - spontaneous and induced. Adv. Lipid Res. 1, 211-252 (1963).

CLARKSON, T. B. Animal models of atherosclerosis. Adv. Vet. Sci. Comp. Med. 16, 151-173 (1972).

CLARKSON, T. B., H. B. LOFLAND, JR., B. C. BULLOCK and H. O. GOODMAN. Genetic control of plasma cholesterol. Studies on squirrel monkeys. Archs. Path. 92, 37-45 (1971).

CLARKSON, T. B., R. W. PRITCHARD, B. C. BULLOCK, N. D. LEHNER, H. B. LOFLAND and R. W. ST. CLAIR. Animal models of atherosclerosis. Animal models for biomedical research 3, 22-41 (1970).

CLIFF, W. J. Biological structure and function of blood vessels. Monograph series. Cambridge University Press. (to be published).

CONNOR, W. E. and D. S. LIN. Origin of milk cholesterol in the rabbit and guinea pig. Am. J. Physiol. 213, 1353-1358 (1967).

CONSTANTINIDES, P. Production of experimental atherosclerosis in animals. J. Atheroscler. Res. 1, 374-385 (1961).

CONSTANTINIDES, P., J. BOOTH and G. CARLSON. Advanced atherosclerosis of the human type in the rabbit. In Drugs affecting lipid metabolism. Editors S. Garattini and R. Paoletti, Elsevier Publishing Co., Amsterdam, The Netherlands, 277-287 (1960).

COOK, R. P., A. KLIMAN and L. F. FIESER. The absorption and metabolism of cholesterol and its main companions in the rabbit - with observations on the atherogenic nature sterols. Archs. Biochem. Biophys. 52, 439-450 (1954).

COOK, R. P. and R. O. THOMSON. The absorption of fat and of cholesterol in the rat, guinea pig and rabbit. Quart. J. exp. Physiol. 36, 61-74 (1951).

DANIELLSON, H., K. EINARRSON and G. JOHANSSON. Effect of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. Eur. J. Biochem. 2, 44-49 (1967).

DAYTON, S. Rationale for the use of lipid-lowering drugs. Fed. Proc. 30, 849-856 (1971).

DEICKE, O. Beobachtungen an Kaninchen mit künstlicher Cholesterinzufuhr. Krankheitsforch 3, 399-418 (1926).

DEUEL, H. J. The Lipids: their chemistry and biochemistry. Vol. II. Biochemistry: digestion, absorption, transport and storage. Interscience Publishers Inc. New York, 259-268 (1955).

DIETSCHY, J. M. and J. D. WILSON. Regulation of cholesterol metabolism (first of three parts). New Engl. J. Med. 282, 1128-1138 (1970a).

DIETSCHY, J. M. and J. D. WILSON. Regulation of cholesterol metabolism (second of three parts) ibid. 1179-1183 (1970b).

DIETSCHY, J. M. and J. D. WILSON. Regulation of cholesterol metabolism (third of three parts) ibid. 1241-1249 (1970c).

DOLE, V. P. and J. T. HAMLIN. Particulate fat in lymph and blood. Physiol. Rev. 42, 674-701 (1962).

DOWLING, R. H. The enterohepatic circulation. Gastroenterology 62, 122-140 (1972).

EADES, C. H. Atherosclerosis. In Annual Reports in medicinal Chemistry. Editor C. K. Cain, Academic Press Inc. New York, 178-188 (1968).

EAPEN, K. J., O. B. GOSWAMI and S. K. PILLAI. Inheritance of serum cholesterol and its relation to body weight in white mice. J. Genet. 60, 222-229 (1971).

EGGEN, D. A. Cholesterol metabolism in rhesus monkey, squirrel monkey and baboon. J. Lipid Res. 15, 139-145 (1974).

EIBL, H. and W. E. M. LANDS. A new, sensitive determination of phosphate. Analyt. Biochem. 30, 51-57 (1969).

EPSTEIN, F. H. Coronary heart disease epidemiology revisited. Clinical and community aspects. Circulation 68, 185-194 (1973).

ESTEP, G. D., R. C. FANGUY and T. M. FERGUSON. The effect of age and heredity upon serum cholesterol levels in chickens. Poultry Sci. 48, 1908-1911 (1969).

EVANS, J. D. and N. OLEKSYSHYN. Observations on the effect of cholesterol and fat supplementation on the

composition of rabbit liver and plasma lipides. Archs. Biochem. Biophys. 85, 317-322 (1959).

FALCONER, D. S. Quantitative inheritance. In Methodology in Mammalian Genetics. W. J. Burdette, Editor. Holden-Day Inc. San Francisco, 193-216 (1963).

FEIGENBAUM, A. S. and E. M. GAMAN. Influence of mother's milk on incidence of spontaneous aortic lesions in weanling rabbits. Proc. Soc. exp. Biol. Med. 124, 1020-1022 (1967).

FELT, V. and P. BENES. Cholesterol esterase (esterifying and hydrolysing) activity in blood serum, liver, kidney and aorta in rabbit atherosclerosis. Enzymol. biol. Clin. 11, 511-520 (1970).

FIDGE, N. H. and C. J. FOXMAN. In vivo transformations of rat plasma very low density lipoprotein into higher density lipoproteins. Aust. J. exp. Biol. med. Sci. 49, 581-593 (1971).

FIESER, L. F. Cholesterol and companions. VII. Steroid dibromides. J. Am. chem. Soc. 75, 5421-5422 (1953).

FILLIOS, L. C. and G. V. MANN. The importance of sex in the variability of the cholesterolaemic response of rabbits fed cholesterol. Circulation Res. 4, 406-412 (1956).

FILLIOS, L. C., R. KAPLAN, R. S. MARTIN and F. J. STARE. Some aspects of the gonadal regulation of cholesterol metabolism. J. Physiol. 193, 47-51 (1958).

FINAGIN, L. K. Role of esterified cholesterol of the liver in bile acid formation. Bull. exp. Biol. Med. 74, 1389-1391 (1972).

FOLCH, J., LEES, M. and G. H. SLOANE STANLEY. A simple method for the isolation and purification of total lipides from animal tissues. J. biol. Chem. 226, 497-509 (1957).

FRASER, R. and F. C. COURTICE. The transport of cholesterol in thoracic duct lymph of animals fed cholesterol with varying triglyceride loads. Aust. J. exp. Biol. med. Sci. 47, 723-732 (1969).

FREDRICKSON, D. S. and R. I. LEVY. Familial hyperlipoproteinaemia. In The metabolic basis of inherited disease. 3rd Edition. J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson, editors. McGraw-Hill, New York, 545-614 (1972).

FRIEDMAN, M. and S. O. BYERS. Effects of diet on serum lipids of fetal, neonatal and pregnant rabbits. Am. J. Physiol. 201, 611-616 (1961).

GARLICK, D. G. and F. C. COURTICE. The composition of the lipoproteins in the plasma of rabbits with hypercholesterolaemia or Triton WR-1339-induced hyperlipaemia. Quart. J. exp. Physiol. 47, 211-220 (1962).

GARLICK, D. G., F. C. COURTICE and M. MUNOZ-MARCUS. Plasma lipoproteins in hyperlipaemic states in man and in the rabbit. Australasian Annals of Medicine 14, 102-110 (1965).

GEISON, R. L. and H. A. WAISMAN. Plasma and tissue cholesterol and lipid levels in rabbits on L-histidine-supplemented diets. Proc. Soc. exp. Biol. Med. 133, 234-237 (1970).

GETZ, G. S., D. VESSELINOVITCH and R. W. WISSLER. A

dynamic pathology of atherosclerosis. Am. J. Med. 46, 657-673 (1969).

GIBBONS, G. F. and K. A. MITROPOULOS. The role of cytochrome P-450 in cholesterol biosynthesis. Eur. J. Biochem. 40, 267-273 (1973).

GIELEN, J., J. Van CANTFORT and J. RENSON. Dosage de la cholésterol-7α-hydroxylase hépatique a l'aide de cholésterol-4-14C. Arch. int. Physiol. Biochem. 76, 581-582 (1968).

GINTER, E. Effect of dietary cholesterol on vitamin C metabolism in laboratory animals. Acta Med. Acad. Sci. Hung. 27, 23-29 (1970).

GINTER, E. Role of ascorbic acid in cholesterol metabolism (literature review). Vopr. Pitan. 31, 25-32 (1972).

GINTER, E. Cholesterol: vitamin C controls its transformation to bile acids. Science 179, 702-704 (1973).

GINTER, E., J. BABALA and E. POLONYOVÁ. Vitamin C und Metabolismus der Lipoide bei mit Atherogener Diät gefütterten Kaninchen. Biologia (Bratislava) 25, 579-586 (1970).

GINTER, E., J. CERVEN, R. NEMEC, and L. MIKUS. Lowered cholesterol catabolism in guinea pigs with chronic ascorbic acid deficiency. Am. J. clin. Nutr. 24, 1238-1245 (1971).

GINTER, E., R. NEMEC, J. CERVEN and L. MIKUS. Quantification of lowered cholesterol oxidation in guinea pigs with latent vitamin C deficiency. Lipids 8, 135-141 (1973).

GLOMSET, J. A. The plasma lecithin: cholesterol acyl-transferase reaction. J. Lipid Res. 9, 155-167 (1968).

GLOMSET, J. A. Plasma cholesterol esterifying enzyme. In Methods in Enzymology. Vol. XV. Steroids and terpenoids. Editor R. B. Clayton, Academic Press Inc. New York, U.S.A., 543-548 (1969).

GLUECK, C. J., R. TSANG, W. BALISTRERI and R. FALLAT. Plasma and dietary cholesterol in infancy: effects of early low or moderate dietary cholesterol intake on subsequent response to increased dietary cholesterol. Metabolism 21, 1181-1192 (1972).

GOFMAN, J. W., F. T. LINDGREN, H. A. ELLIOT, W. MANTZ, J. HEWITT, B. STRISOWER, V. HERRING and T. P. LYON. Role of lipids and lipoproteins in atherosclerosis. Science III, 166-171 (1950).

GOODMAN, De W. S. Cholesterol ester metabolism. Physiol. Rev. 45, 747-839 (1965).

GOODMAN, De W. S. Hydrolysis and formation of cholesterol esters in rat liver. In Methods in Enzymology, Vol. XV. Steroids and terpenoids. Editor R. B. Clayton, Academic Press Inc., New York, U.S.A., 522-537 (1969).

GOULD, R. G. and C. B. TAYLOR. Effect of dietary cholesterol on hepatic cholesterol synthesis. Fed. Proc. 9, 179 (1950).

GOULD, R. G. and R. P. COOK. The metabolism of cholesterol and other sterols in the animal organism. In Cholesterol. Editor R. P. Cook, Academic Press Inc., New York, U.S.A., 245-252 (1958).

GRASSHOF, H. Die Überführung von Koprostanon in Koprosterin. Hoppe Seyler's z. physiol. Chem. 225, 197-198 (1934a).

GRASSHOF, H. Über Cholestenon und Koprostanon. Hoppe Seyler's z. physiol. Chem. 223, 249-258 (1934b).

GRUNDY, S. M. and E. H. AHRENS, Measurement of cholesterol turnover, synthesis, and absorption in man, carried out by isotope kinetic and sterol balance methods. J. Lipid Res. 10, 91-107 (1969).

GRUNDY, S. M., E. H. AHRENS and T. A. MIETTINEN. Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. J. Lipid Res. 6, 397-410 (1965).

HAMPRECHT, B., C. NÜSSLER, G. WALTINGER and F. LYNEN. Influence of bile acids on the activity of rat liver 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. 1. Effect of bile acids *in vitro* and *in vivo*. Eur. J. Biochem. 18, 10-14 (1971).

HAMPRECHT, B., R. ROSCHER, G. WALTINGER and C. NÜSSLER. Influence of bile acids on the activity of rat liver 3-hydroxy-3-methyl-glutaryl coenzyme A reductase. 2. Effect of cholic acid in lymph fistula rats. Eur. J. Biochem. 18, 15-19 (1971).

HARRIS, K. L. and J. M. FELTS. Kinetics of chylomicron triglyceride removal from plasma in rats: a comparison of the anaesthetized and unanaesthetized states. J. Lipid Res. 11, 75-81 (1970).

HARRIS, L. J., B. J. CONSTABLE, A. N. HOWARD and A. LEADER. Vitamin C economy of rabbits. Br. J. Nutr. 10, 373-382 (1956).

HARRIS, R. A., J. E. MACNINTCH and F. W. QUACKENBUSH. Mechanism of suckling rat hypercholesterolaemia: dietary and drug studies. J. Nutr. 90, 40-46 (1966).

HASHIMOTO, S. and S. DAYTON. Cholesterol esterifying activity in serum of the cholesterol-fed rabbit. Proc. Soc. exp. Biol. Med. Sci. 137, 1186-1188 (1971).

HAVEL, R. J., H. A. EDER and J. H. BRAGDON. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. clin. Invest. 34, 1345-1353 (1955).

HELLSTRÖM, K. On the bile acid and neutral fecal steroid excretion in man and rabbits following cholesterol feeding. Bile acids and steroids 150. Acta Physiol. Scand. 63, 21-35 (1965).

HELLSTRÖM, K. and J. SJÖVALL. Turnover of deoxycholic acid in the rabbit. J. Lipid Res. 3, 397-404 (1962).

HENBEST, H. B. and E. R. H. JONES. Studies in the sterol group. Part XLIX. 7-substituted cholesterol derivatives and their stereochemistry (Part II). Esters of the epimeric 7-hydroxycholesterols. J. Chem. Soc. II, 1792-1797 (1948).

HICKMAN, P. E., B. J. HORTON and J. R. SABINE. Effect of adrenalectomy on the diurnal variation of hepatic cholesterogenesis in the rat. J. Lipid Res. 13, 17-22 (1972).

HIGGINS, M., T. KAWACHI and H. RUDNEY. The mechanism of the diurnal variation of hepatic HMG-CoA reductase activity in the rat. Biochem. biophys. Res. Commun. 45, 138-144 (1971).

HIRATA, K., F. WADA and Y. SAKAMOTO. Cholesterol synthesis and electron transport system in liver microsomes. J. Japan. Biochem. Soc. 39, 753 (1967).

HO, K. J. and C. B. TAYLOR. Control mechanisms of cholesterol biosynthesis. Archs. Path. 90, 82-92 (1970).

HORTON, B. J., P. E. HICKMAN and J. R. SABINE. The effect of diet on the diurnal variation of cholesterol synthesis in rat liver. Life Sci. 9 II, 1409-1417 (1970).

HUBER, J., W. GUDER, S. LATZIN and B. HAMPRECHT. The influence of insulin and glucagon on hydroxymethylglutaryl coenzyme A reductase activity in rat liver. Hoppe-Seyler's z. physiol. Chem. 354, 795-798 (1973).

IGNATOWSKI, A. Über die Wirkung des tierischen Eiweisses auf die Aorta and die parenchymatosen Organe der Kaninchen. Virchows Arch. path. Anat. Physiol. 198, 248-270 (1909).

IMAI, Y. and H. MATSUMURA. Genetic studies on induced and spontaneous hypercholesterolaemia in rats. Atherosclerosis 18, 59-64 (1973).

KANDUTSCH, A. A. and S. E. SAUCIER. Prevention of cyclic and triton-induced increases in hydroxymethylglutaryl coenzyme A reductase and sterol synthesis by puromycin. J. biol. Chem. 244, 2299-2305 (1969).

KATO, R. and A. TAKAHASHI. Thyroid hormone and activities of drug-metabolizing enzymes and electron transport systems of rat liver microsomes. Mol. Pharmacol. 4, 109-120 (1968).

KEVERLING BUISMAN, J. A., W. STEVENS and J. v.d. VLIET. Investigations on sterols. I. A new synthesis of 7-dehydrocholesterol (provitamin D). Rec. Trav. chim. Pays-Bas Belg. 66, 83-92 (1947).

KRITCHEVSKY, D. Experimental atherosclerosis in primates and other species. Ann. N.Y. Acad. Sci. 162, 80-88 (1969).

KRITCHEVSKY, D., A. M. MARCUCCU, P. SALLATA and S. A. TEPPER. Comparison of amorphous and crystalline cholesterol in establishment of atherosclerosis in rabbits. Med. exp. 19, 185-193 (1969).

KRITCHEVSKY, D., P. SALLATA AND S. A. TEPPER. Experimental atherosclerosis in rabbits fed cholesterol-free diets. Part 2. Influence of various carbohydrates. J. Atheroscler. Res. 8, 697-703 (1968).

KRITCHEVSKY, D. and S. A. TEPPER. Experimental atherosclerosis in rabbits fed cholesterol-free diets: influence of chow components. J. Atheroscler. Res. 8, 357-369 (1968).

KRITCHEVSKY, D., S. A. TEPPER and J. A. STORY. Influence of vitamin C on hydroxylation and side chain oxidation of cholesterol in vitro. Lipids 8, 482-484 (1973).

KRUSKI, A. W. and K. A. NARAYAN. Lipoprotein synthesis in chickens fed cholesterol. Atherosclerosis 15, 141-145 (1972).

LACKO, A. G., H. L. RUTENBERG and L. A. SOLOFF. Serum cholesterol esterification in species resistant and susceptible to atherosclerosis. Atherosclerosis 19, 297-305 (1974).

LAIRD, C. W. and R. R. FOX. The effect of age on cholesterol and PBI levels in the III/IIIc hybrid rabbits. Life Sciences 9, II, 1243-1253 (1970).

LAIRD, C. W., R. R. FOX, H. S. SCHULTZ, B. P. MITCHELL and E. M. BLAU. Strain variations in rabbits: biochemical indicators of thyroid functions. Life Sciences 9, II, 203-214 (1970).

LEVY, R. I. and D. S. FREDRICKSON. Heterogeneity of plasma high density lipoproteins. J. clin. Invest. 44, 426-441 (1965).

LEWIS, B. Metabolism of plasma lipoproteins. In Scientific Basis of Medicine Annual Reviews. Editors I. Gilliland and J. Francis. 118-144 (1972).

LINDGREN, F. T., H. A. ELLIOT and J. W. GOFMAN. The ultracentrifugal characterisation and isolation of human blood lipids and lipoproteins, with applications to the study of atherosclerosis. J. Phys. and Colloid Chem. 55, 80-93 (1951).

LOFLAND, H. B., T. B. CLARKSON, R. W. St. CLAIR and N. D. M. LEHNER. Studies on the regulation of plasma cholesterol levels in squirrel monkeys of two genotypes. J. Lipid Res. 13, 39-47 (1972).

LOPEZ-S, A. Serum lipid transport systems: recent advances. Lipids 6, 369-377 (1971).

LOSSOW, W. J., S. N. SHAH and I. L. CHAIKOFF. Isolation of cholesterol esterifying activity in rat serum by ultracentrifugal flotation. Biochim. biophys. Acta 116, 172-174 (1966).

MANN, G. V. A method for measurement of cholesterol in blood serum. Clin. Chem. 7, 275-284 (1961).

MATHE, D., F. D. HOLLANDER and F. CHEVALLIER. In vivo studies of the possible role of cholesteryl esters as precursors of bile acids. Biochimie 54, 1479-1481 (1972).

MAYER, D., F. W. KOSS and A. GLASENAPP. Bestimmung der Cholesterin -7α-hydroxylase - Aktivität in der Rattenleber. Hoppe-Syler's z. Physiol. Chem. 353, 921-932 (1972).

McINTYRE, N., K. J. ISSELBACKER. Role of the small intestine in cholesterol metabolism. Am. J. clin. Nutr. 26, 647-656 (1973).

McINTYRE, N., K. KIRSCH, J. C. ORR and K. J. ISSELBACKER. Sterols in the small intestine of the rat, guinea pig and rabbit. J. Lipid Res. 12, 336-346 (1971).

MEEKER, D. R. and H. D. KESTEN. Effect of high protein diets on experimental atherosclerosis of rabbits. Archs. Path. 31, 147-162 (1941).

MIETTINEN, T. A., E. H. AHRENS and S. M. GRUNDY. Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. J. Lipid Res. 6, 411-424 (1965).

MILLS, G. L. and C. E. TAYLAUR. The distribution and composition of serum lipoproteins in eighteen animals. Comp. Biochem. Physiol. 40B, 489-501 (1971).

MINARI, O. and D. B. ZILVERSMIT. Use of KCN for stabilization of colour in direct Nesslerization of Kjeldahl digests. Analyt. Biochem. 6, 320-327 (1963).

MITANI, F. and S. HORIE. Studies on P-450. V. On the substrate-induced spectral change of P-450 solubilised from bovine adrenocortical mitochondria. J. Biochem. (Tokyo) 65, 269-280 (1969).

MITROPOULOS, K. A. and S. BALASUBRAMANIAM. Cholesterol 7α-hydroxylase in rat liver microsomal preparations. Biochem. J. 128, 1-9 (1972).

MITTON, J. R., N. A. SCHOLAN and G. S. BOYD. The oxidation of cholesterol in rat liver sub-cellular particles. The cholesterol-7α-hydroxylase enzyme system. Eur. J. Biochem. 20, 569-579 (1971).

MORRIS, J. N. and M. J. GARDNER. Epidemiology of ischaemic heart disease. Am. J. Med. 46, 674-683 (1969).

MUKHERJEE, T. K., G. W. FRIARS and J. D. SUMMERS. Estimates of changes in plasma cholesterol and protein in relation to certain reproductive traits in female breeder turkeys. Poult. Sci. 48, 2081-2086 (1969).

MYANT, N. B. Hormonal control of cholesterol metabolism. Biol. Basis of Med. 2, 193-220 (1968).

NATIONAL INSTITUTES OF HEALTH. Arteriosclerosis - a report by the National Heart and Lung Institute task force on arteriosclerosis, Vol. 1. DHEW Publication No. (NIH) 72-137, Washington, D.C. 12-16 (1971).

NESTEL, P. J. Cholesterol turnover in man. Adv. Lipid Res. 8, 1-39 (1970).

NOVITSKII, A. A. Mechanism likely to cause resistance to alimentary hypercholesterolaemia in rabbits. Cor Vasa 13, 280-290 (1971).

O'KELLY, J. C. Seasonal variations in the plasma lipids of genetically different types of cattle: grazing steers. Comp. Biochem. Phys. 43B, 283-294 (1972).

O'KELLY, J. C. Seasonal variations in the plasma lipids of genetically different types of cattle: steers on different diets. Comp. Biochem. Phys. 44A, 303-312 (1973).

OMURA, T. and R. SATO. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. biol. Chem. 239, 2370-2378 (1964).

PATTON, N. M., R. V. BROWN and C. C. MIDDLETON. Familial cholesterolaemia in pigeons. Atherosclerosis 19, 307-314 (1974).

PEETERS, H. The apoprotein and lipid moieties of the lipoproteins. In Protides of the Biological Fluids 18, Editor H. Peeters, Pergamon Press, U.K., 3-26 (1970).

PERTSEMLIDIS, D., E. H. KIRCHMAN and A. H. AHRENS. Regulation of cholesterol metabolism in the dog. I. Effects of complete bile diversion and of cholesterol feeding on absorption, synthesis accumulation and excretion rates measured during life. J. clin. Invest. 52, 2353-2367 (1973).

POLLAK, O. J. Correlation between chemical and morphologic alterations in experimental atherosclerosis. Archs. Path. 39, 11-15 (1945).

POLLAK, O. J. Purina rabbit chow as a variant of experimental atherosclerosis. J. Atheroscler. Res. 5, 524-525 (1965).

POLLAK, O. J. Successful prevention of experimental hypercholesterolaemia and cholesterol atherosclerosis in the rabbit. Circulation 7, 696-701 (1953).

POMARE, E. W. and K. W. HEATON. Alteration of bile salt metabolism by dietary fibre (Bran). Br. Med. J. 4, 262-264 (1973).

POOL, W. R., H. L. NEWMARK, C. DALTON, R. F. BANZIGER and A. N. HOWARD. Effect of biotin and ascorbic acid on the development of atherosclerosis in rabbits. Atherosclerosis 14, 131-135 (1971).

PRIOR, J. T., D. M. KURTZ and D. D. ZIEGLER. The hypercholesteremic rabbit. Archs. Path. 71, 672-684 (1961).

QUINTAO, E., S. M. GRUNDY and E. H. AHRENS. Effects of dietary cholesterol on the regulation of total body cholesterol in man. J. Lipid Res. 12, 233-247 (1971).

RAUTUREAU, J., T. COSTE and M. RAUTUREAU. Métabolisme normal du cholestérol (I^{re} partie). Arch. Fr. Mal. App. Dig. 61, 209-224 (1972).

RAZ, A., F. A. KUMMEROW and T. NISHIDA. Various factors affecting cholesterol esterification in plasma lipoproteins by lecithin-cholesterol acyltransferase. Biochem. Biophys. Acta 176, 591-599 (1969).

REDGRAVE, T. G. Cholesterol feeding alters the metabolism of thoracic-duct lymph lipoprotein cholesterol in rabbits but not in rats. Biochem. J. 136, 109-113 (1973).

REDGRAVE, T. G. Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. J. clin. Invest. 49, 465-471 (1970).

REDGRAVE, T. G. and C. E. WEST. Differential effect of piperazine on cholesterol metabolism in male and female rabbits. Aust. J. exp. Biol. med. Sci. 50, 153-164 (1972).

REISER, R. Control of adult serum cholesterol by the nutrition of the suckling: a progress report. Circulation 44, 11-3 (1971).

REISER, R. and Z. SIDELMAN. Control of serum cholesterol homeostasis by cholesterol in the milk of the suckling rat. J. Nutr. 102, 1009-1016 (1972).

ROGERS, E. W. Excretion of piperazine salts in urine. Br. Med. J. 1, 136-137 (1958).

ROHRSCHNEIDER, W. Beitrag zur Kenntnis der experimentellen Hypercholesterinamie des Kaninchens. Virchow's Arch. path. Anat. Physiol. 256, 139-149 (1925).

ROSE, G. Environmental and host factors in coronary heart disease, including risk factors: an epidemiological view. Current developments in Europe. In Proceedings of the second international symposium on atherosclerosis. Editor R. J. Jones, Springer Verlag, New York, Inc. 310-314 (1969).

ROSE, H. G. Origin of cholesteryl esters in the blood of cholesterol-fed rabbits: relative contributions of serum lecithin-cholesterol acyltransferase and hepatic ester synthesis. Biochim. Biophys. Acta 260, 312-326 (1972).

ROSENMAN, R. H. and M. FRIEDMAN. The possible role of behaviour patterns in proneness and immunity to coronary heart disease. In Coronary heart disease, Editors

H. I. Russek and B. L. Zohman. J. B. Lippincott Co. Phil., U.S.A., 77-84 (1971).

SAITO, S. and L. C. FILLIOS. Certain aspects of cholesterol metabolism and hepatic protein synthesis in the rat. Am. J. Physiol. 207, 1287-1294 (1964).

SALTYKOW, S. Die experimentell erzeugten Arterienveränderungen in ihrer Beziehung zu Atherosklerose und verwandten Krankheiten des Menschen. Zentbl. allg. Path. path. Anat. 19, 321-398 (1908).

SALVADOR, R. A., S. HABER, C. ATKINS, B. W. GOMMI and R. M. WELCH. Effect of Clofibrate and 1-methyl-4 piperidyl bis (p-chloro-phenoxy) acetate (Sandoz 42-348) on steroid and drug metabolism by rat liver microsomes. Life Sciences 9, II, 397-407 (1970).

SCHAEFFER, L. E., D. ADLERSBERG and A. G. STEINBERG. Heredity, environment and serum cholesterol. Circulation 17, 537-542 (1958).

SCHAFFERT, R. R. and G. R. KINGSLEY. A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. J. biol. Chem. 212, 59-68 (1955).

SCHOENFIELD, L. J., G. G. BONORRIS and P. GANZ. Induced alterations in the rate-limiting enzymes of hepatic cholesterol and bile acid synthesis in the hamster. J. Lab. Clin. Med. 82, 858-869 (1973).

SCHOLAN, N. A. and G. S. BOYDE. The cholesterol 7α-hydroxylase enzyme system. Hoppe-Seyler's Z. Physiol. Chem. 349, 1628-1630 (1968).

SCHUMAKER, V. N. and G. H. ADAMS. Circulating lipoproteins. An. Rev. Biochem. 38, 113-136 (1969).

SHEFER, S., S. HAUSER and E. H. MOSBACH. 7α-hydroxylation of cholestanol by rat liver microsomes. J. lipid Res. 9, 328-333 (1968).

SHOPE, R. E. The effect of age on the total and combined cholesterol of the blood serum. J. biol. Chem. 80, 141-148 (1928).

SHORE, V. G., B. SHORE and R. G. HART. Changes in apolipoproteins and properties of rabbit very low density lipoproteins on induction of cholesteremia. Biochemistry 13, 1579-1585 (1974).

SIDELMAN, Z. and R. REISER. Dietary cholesterol during early development and its effects on serum cholesterol concentration of adult rats. Fedn. Proc. Fedn. Am. Socs. exp. Biol. 30, A-348 (1971).

SIMMONDS, W. J. Effect of bile salts on the rate of fat absorption. Am. J. clin. Nutr. 22, 266-272 (1969).

SINITSINA, T. A. and T. H. LOVYAGINA. Variation of experimental hypercholesterolaemia in rabbits. Dokl. Akad. Nauk. SSSR 110, 1126-1129 (1956).

SLAKEY, L. L., M. C. CRAIG, E. BEYTIA, A. BRIEDIS, D. H. FELDBRUGGE, R. E. DUGAN, A. A. QURESHI, C. SUBBARAYAN and J. W. PORTER. The effects of fasting, refeeding, and time of day on the levels of enzymes effecting the conversion of β-hydroxy-β-methylglutaryl-coenzyme A to squalene. J. biol. Chem. 247, 3014-3022 (1972).

SNEDECOR, G. W. and W. G. COCHRAN. Statistical methods 6th edition. The Iowa State University Press. Ames, Iowa, U.S.A. p.86 (1967).

SODHI, H. W. and B. J. KUDCHODKAR. Correlating metabolism of plasma and tissue cholesterol with that of plasma lipoproteins. Lancet 1, 513-519 (1973).

SPERRY, W. M. and M. WEBB. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 187, 97-106 (1950).

SPITTLE, C. R. Atherosclerosis and Vitamin C. Lancet 2, 1280-1281 (1971).

STOUT, C. and M. E. GROOVER. Spontaneous versus experimental atherosclerosis. Ann. N.Y. Acad. Sci., 162, 89-98 (1969).

STRONG, J. P. and D. A. EGGEN. Risk factors and atherosclerotic lesions. In Proceedings of the second international symposium on atherosclerosis. Editor R. J. Jones. Springer Verlag, New York Inc. 355-364 (1969).

STUFFLEBEAN, C. E. and J. F. LASLEY. Hereditary basis of serum cholesterol level in beef cattle. J. Hered. 60, 15-16 (1969).

SWELL, L. and C. R. TREADWELL. Enzymic preparation of labeled unsaturated fatty acid esters of cholesterol. Analyt. Biochem. 4, 335-340 (1962).

TAYLOR, C. B. and K. J. HO. A review of human cholesterol metabolism. Archs. Path. 84, 3-14 (1967).

THOLLDTE, M. Hypercholesterinämie, Blutdruck und Gefäfsveränderungen im Tierversuch. Beitr. pathol.

Anat. 77, 61-120 (1927).

TRULZSCH, D., H. GREIM, P. CZYGAN, F. HUTTERER, F. SCHAFFNER, H. POPPER, D. Y. COOPER and O. ROSENTHAL. Cytochrome P-450 in 7α-hydroxylation of taurodeoxycholic acid. Biochemistry 12, 76-79 (1973).

TUMBLESON, M. E. and D. P. HUTCHESON. Age related serum cholesterol, glucose and total bilirubin concentrations of female dairy cattle. Proc. Soc. exp. Biol. Med. 138, 1083-1085 (1971).

TURNER, K. B. and E. H. BIDWELL. Some effects of iodine given to rabbits after a period of cholesterol feeding. Proc. Soc. exp. Biol. Med. 35, 656-660 (1937).

TURNER, K. B., C. H. PRESENT and E. H. BIDWELL. The role of the thyroid in the regulation of the blood cholesterol of rabbits. J. exp. Med. 67, 111-127 (1938).

URIEL, J. and P. GRABAR. Emploi de colorants dans l'analyse électrophorétique et immuno électrophorétique en milieu gélifié. Annls. Inst. Pasteur 90, 427-440 (1956).

VAN BELLE, H. Cholesterol, bile acids and atherosclerosis. A biochemical review. North Holland Publishing Co., Amsterdam, p.83 (1965).

VANDENHEUVAL, F. A. and S. A. COURT. Reference high efficiency nonpolar packed columns for the gas-liquid chromatography of nanogram amounts of steroids. Part 1. Retention time data. J. Chromatog. 38, 439-459 (1968).

WADA, F., K. HIRATA, K. NAKAO and Y. SAKAMOTO. Participation of P-450 in 7α-hydroxylation of cholesterol. J. Biochem. (Tokyo) 64, 415-417 (1968).

WADA, F., K. HIRATA, and Y. SAKAMOTO. Possible participation of cytochrome P-450 in cholesterol synthesis. J. Biochem. (Tokyo) 65, 171-175 (1969).

WAGNER, W. D. and T. B. CLARKSON. Mechanisms of the genetic control of plasma cholesterol in selected lines of show racer pigeons. Proc. Soc. exp. Biol. Med. 145, 1050-1057 (1974).

WALTON, K. W. The validity of the lipid-fed rabbit as a model for the study of atherogenesis. J. Pathol. 109, ii (1973).

WANG, C. I., L. E. SCHAEFER, S. R. DRACHMAN and D. ADLERSBERG. Plasma lipid partition of the normal and cholesterol-fed rabbit. J. Mt. Sinai Hosp. 20, 19-25 (1953).

WATT, S. M. and SIMMONDS, W. J. Uptake and efflux by everted intestinal sacs of micellar cholesterol in bile salts and non-ionic detergent. Biochem. Biophys. Acta 225, 347-355 (1970).

WEIBUST, R. S. Inheritance of plasma cholesterol levels in mice. Genetics 73, 303-312 (1973).

WERNER, E. A. The constitution of carbamides. Part IX. The interaction of nitrous acid and mono-substituted ureas. The preparation of diazomethane, diazoethane, diazo-n-butane and diazo-isopentane from the respective nitrosoureas. J. Chem. Soc. 115, 1093-1102 (1919).

WEST, C. E. and T. G. REDGRAVE. Reservations on the use of polyunsaturated fats in human nutrition. Search 5, 90-94 (1974).

WILLIAMS, C. H., D. J. DAVID and O. IISMAA. The determination of chromic oxide in faeces samples by atomic absorption spectrophotometry. J. agric. Sci. 59, 381-385 (1962).

WILSON, J. D. The quantitation of cholesterol excretion and degradation in the isotopic steady state in the rat: the influence of dietary cholesterol. J. Lipid Res. 5, 409-417 (1964).

ZAITSEV, V. F., L. A. MYASNIKOV, I. V. KASATKINA, N. M. LOBOVA and T. I. SUKASOVA. The effect of ascorbic acid on experimental atherosclerosis. Cor Vasa 1, 19-25 (1964).

ZILVERSMIT, D. B. The composition and structure of lymph chylomicrons in dog, rat and man. J. clin. Invest. 44, 1610-1622 (1965).

ZILVERSMIT, D. B., L. B. HUGHES and M. REMINGTON. Hypolipidemic effect of pregnancy in the rabbit. J. Lipid Res. 13, 750-756 (1972).

ZLATKIS, A. and B. ZAK. Study of a new cholesterol reagent. Analyt. Biochem. 29, 143-148 (1969).